Studies on the N-dealkylation of codeine by Candida tropicalis

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STUDIES ON THE N-DEALKYLATION OF CODEINE BY *Candida tropicalis*

Submitted by Amit Makwana, B.Sc.(Hons)

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This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of C.J. Soper, B.Pharm., M.Sc., Ph.D., M.R.Pharm.S. and S.K. Branch, B.Pharm., Ph.D., M.R.Pharm.S.

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Nothing in the world can take the
place of persistance.
Talent will not; nothing is more common
than unsuccessful men with talent.
Genius will not; unrewarded genius is
almost a proverb.
Education will not; the world is full
of educated derelicts.
Persistance and determination alone
are omnipotent.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to both Dr. Colin J. Soper and Dr. Sarah K. Branch for their guidance, advice and encouragement throughout the course of these studies. I am also indebted to Dr. Richard England of the School of Chemical Engineering, University of Bath, for the loan of laboratory fermenters and his helpful advice relating to this equipment.

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I am also grateful to the University of Bath Research Fund for providing the essential financial support.

Finally, I would like to thank Sharmila for proof-reading and especially for her encouragement, moral support and for giving me endless views of the M4!

DEDICATION

This thesis is dedicated to wife Sharmila and my parents for their unfailing support and strength and to my parents not present for the wisdom and courage which they have undoubtly passed on.

SUMMARY

In the general introduction, the importance of N-dealkylation in pharmaceutical studies is disscussed. The scope and potential advantages of microbial transformations in effecting N-dealkylation are summarised. The genus *Candida*, the cytochrome P-450 of yeasts and the mechanism of cytochrome P-450 function are discussed.

Chapter 2 describes the screening of selected *Candida tropicalis* strains for their ability to N-dealkylate the alkaloid codeine. The development of techniques for microbial growth determination and qualitative analytical techniques are also detailed.

Chapter 3 describes the development of a growth medium for the microorganism selected for further study, *Candida tropicalis* ATCC 32113. The effect of growth medium components on codeine transformation is investigated and parameters which may influence microbial transformation, such as substrate concentration, inoculum volume and vitamins, are optimized. Cytochrome P-450 is proposed as the enzyme responsible for the codeine N-demethylation reaction.

Chapter 4 discusses the use of a laboratory 7 litre batch fermenter. The effect of carbon sources, pH, agitation and oxygen in the medium are examined to optimize codeine N-demethylation and cytochrome P-450 yield.

Chapter 5 describes the development of procedures for the preparation of a cell free extract from *Candida tropicalis* ATCC 32113. Procedures for further purification of the cell free extracts are discussed. Conditions for the N-demethylation of codeine by purified cell free extracts in the reaction mixture are optimized in terms of substrate concentration and co-factor requirement. The effect of specific enzyme inhibitors are investigated. A reaction mechanism for the N-demethylation of codeine by cell free extracts of *Candida tropicalis* ATCC 32113 is proposed. The stability of the cytochrome P-450 enzyme is examined.

In the discussion the data is considered with reference to current microbial transformation literature. Comparisons are also made with mammalian biotransformations of similar substrates and suggestions for future study in specific areas are emphasised.

ABBREVIATIONS

BSA	Bovine Serum Albumin
^{13}C	Carbon-13
GC	Gas Ch romatography
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
id	Internal Diameter
Km	Michaelis Constant
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
t_r	Retention time
TLC	Thin Layer Chromatography
U V	Ultra Violet
V_{max}	Maximum Velocity

CONTENTS

CHAPTER 2. SCREENING OF *Candida tropicalis* STRAINS FOR N-DEMETHYLATION ACTIVITY

ORIGIN AND SCOPE

Medicinal chemists have previously experienced difficulties in effecting N-dealkylation reactions by direct chemical methods in the preparation of useful drugs and drug intermediates. Studies carried out at the University of Bath have suggested that N-dealkylation procedures using microbial enzymes may provide a safe and more efficient alternative to the chemical methods. Subsequently a microbial transformation system has been developed, where several species of the fungus *Cunninghamella* are able to N-demethylate a wide variety of drug molecules. These studies have investigated the involvement of cytochrome P-450 N-demethylation by *Cunninghamella* sp. and analytical techniques have been developed to enable detailed examination of the transformation process. Difficulties have arisen with the use of *Cunninghamella* sp. due to its growth characterisics in fermenters. This has prompted the search for other N-demethylating microorganisms and the studies reported in this thesis are concerned with an investigation of one such organism, the pathogenic yeast *Candida*.

It was intended that the cytochrome P-450 in *Candida* sp. would be characterised with respect to N-demethylation activity using codeine as the substrate. The analytical techniques used previously would be adapted for these characterisation studies. The cytochrome P-450 protein(s) would then be purified whilst maintaining and hopefully optimising the N-demethylation activity. This would facilitate detailed studies of the yeast cytochrome P-450 protein(s) and its interaction with ergosterol biosynthesis inhibitors.

CHAPTER ONE

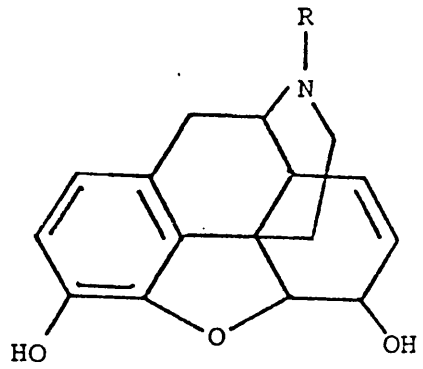
GENERAL INTRODUCTION

1. INTRODUCTION

1.1 Pharmacological importance of N-alkyl functions

N-dealkylation involves the cleavage of an N-alkyl bond resulting in removal of the alkyl function from the parent compound and its replacement with a hydrogen atom. The N-alkyl functions are present in a variety of drug molecules, usually in saturated cyclic structures or alkylamine chains where the N-substituent has a marked effect on the pharmacological activity of the drug. For example, in the 1,4-benzodiazepine tranquilizers a change from N-methyl in diazepam to N-diethylaminoethyl in flurazepam results in an increase in the plasma half life and duration of action of the drug (Hollister, 1982). Structure activity studies of the morphine like analgesics have also shown that the N-substituent significantly influences the analgesic activity of the molecule (Green *et al*, 1954; Beckett, 1956). The naturally occuring alkaloid morphine is isolated from the seeds of *Papaver somniferum* and possesses an N-methyl tertiary nitrogen substituent. Small *et al* (1938) attempted to increase the analgesic potency and decrease the physical dependance liability of morphine by studying the effect of modifications to the peripheral groups of the morphine molecule. The changes in the N-substituent significantly affected analgesic activity and the pharmacological response. The results obtained by Small *et al* are summarised in Table 1.1.

The outstanding observation in Table 1.1 is the 14 fold increase in analgesic potency resulting from the presence of an N-phenethyl



N-substituent	Analgesic activity*
$-CH_3$ (morphine)	100
-H (normorphine)	5
$-CH_2CH_2-C_6H_5$	1400
propyl, isobutyl, allyl, methallyl	morphine antagonists which also possess variable analgesic activity.

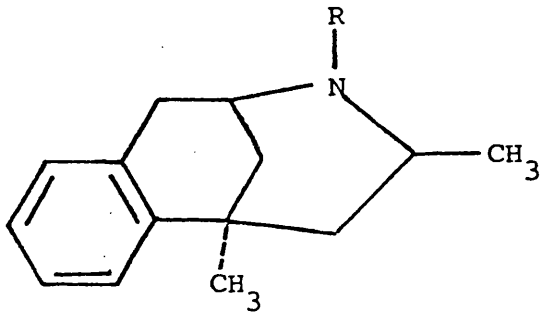
* is expressed as percent ratio of the ED_{50} obtained in mouse hot plate tests for morphine to that of the modified congener, where ED_{50} is the dose required to effect a measured pharmacological response in 50% of the animals tested.

Table 1·1 Effect of N-substituent variation on the pharmacological activity of the morphine molecule.

substituent. These studies also showed that replacement of the N-methyl group with larger alkyl or allyl functions confers morphine anatagonistic properties on the molecule. These findings led Gilbert and Martin (1976) to speculate that several types of opiate receptors may be involved in the morphine response mechanism. It was proposed that morphine exhibited agonist activity at one type of receptor (termed u) whereas compounds such as nalorphine (N substituent = $-CH_2\ CH{=}CH_2$) exhibited antagonist activity at the u receptor with concurrent agonist activity at a second analgesic receptor site termed k (Gilbert and Martin, 1976).

A beneficial feature of the morphine antagonist analgesics is the lower physical dependance exhibited by these compounds (Kotick *et al*, 1980). Further attempts to separate the analgesic and dependance characteristics of morphine-like compounds were stimulated by these findings: for example, the production of buprenorphine, a tertiary alcohol derived from the Diels-Alder adduct of thebaine with an N-cyclopropyl methyl substituent. These modifications produced mixed agonist and antagonist characteristics which confer on buprenorphine the favourable properties of strong analgesia in the absence of physical dependance or psychomimetic effects (Ward *et al*, 1977).

Differences in pharmacological activity have also been observed with various N-substituted benzomorphan derivatives. The pharmacological data obtained for the more potent 3,5-dimethyl isomers are shown in Table 1.2. The results showed that the N-cyclopropylmethyl derivative appeared to be free of physical dependence characteristics whilst exhibiting the highest analgesic activity. N-alkyl functions therefore are present in a wide variety



N-substituent	Analgesic activity*
$-CH_3$	11·9
$-CH_2CH=C(CH_3)_2$	**
$-CH_2$	2·2

* Expressed as ED_{50} obtained in mouse hot-plate tests

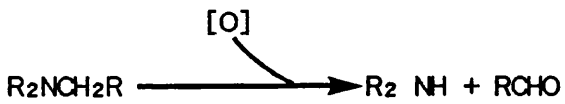
** Inactive at sub-toxic dose

Table 1·2 Analgesic activity of some 3-methyl 6,7-benzomorphans

of drug molecules. In the design of a new drug it is often required to remove the N-alkyl function from the parent drug to form the nor intermediate and replace it with different n-substituents. This enables relationships to be established between the chemical structures of a series of drugs and pharmacological activity. With this acquired knowledge it is possible to predict and design drugs with specific biological properites.

1.2 N-dealkylation in Drug Metabolism

The dealkylation reaction is now recognised as a major pathway in drug metabolism in mammalian systems (McMahon, 1966) and has the following stoichiometry:



Oxidative N-, O- and S- dealkylation of many drugs and foreign compounds is effected by enzyme systems located in the microsomal fraction of the mammalian liver and,to a lesser extent, in the kidneys, lungs and brain (Sato and Omura, 1978). The hepatic enzyme systems catalyse reactions in a wide range of substrates and the N-dealkylation of both tertiary and secondary amines has been demonstrated (Nerbert *et al*, 1981; Gram, 1971). The N-dealkylation reaction is a major component in the *in vivo* mammalian metabolism of a range of drugs including codeine and morphine (Adler, 1954; Misra*et al*, 1961). The liver enzymes may be divided into two groups. The

first group are responsible for general detoxification of drugs and xenobiotics to facilitate excretion from the body. They are also involved in the biosynthesis of endogenous compounds such as steroid hormones and prostaglandins. The second group consists of enzymes inducible with narrow specificity and inducable enzymes (Wiseman and King, 1982) for more effective metabolism of foreign compounds entering the body. N-dealkylation, therefore, does not occur in isolation but is an intermediate step in an overall drug metabolic pathway at the end of which many metabolites are conjugated with glucuronic acid prior to excretion (Klingele, 1972). An example of this is illustrated in Figure 1.1 with the mammalian metabolism of chlorpromazine (Wilson, 1971), where N-demethylation, S-oxidation and hydroxylation all occur concurrently before conjugation and excretion. Adler and Latham (1950) first showed with *in vitro* and *in vivo* mammalian studies that codeine is O-demethylated to produce morphine and formaldehyde, and oxidation of the N-methyl group produces norcodeine (Figure 1.2). Studies with amines have shown that N-demethylation is more rapid with secondary amines than with tertiary amines, and the rate of N-delkylation decreases with increased chain length of the N-alkyl substituent (Gram, 1971).

The hepatic enzymes responsible for drug metabolism are now collectively referred to as cytochrome P-450 monooxygenases (P-450). The cytochrome P-450 term refers to a pigment with peak absorbance at 450nm in the reduced carbon monoxide (CO) difference spectrum (Omura and Sato, 1964). They are called monooxygenases because they activate molecular oxygen resulting in incorporation of an oxygen atom into the substrate molecule (Capdevila *et al*, 1984). Associated

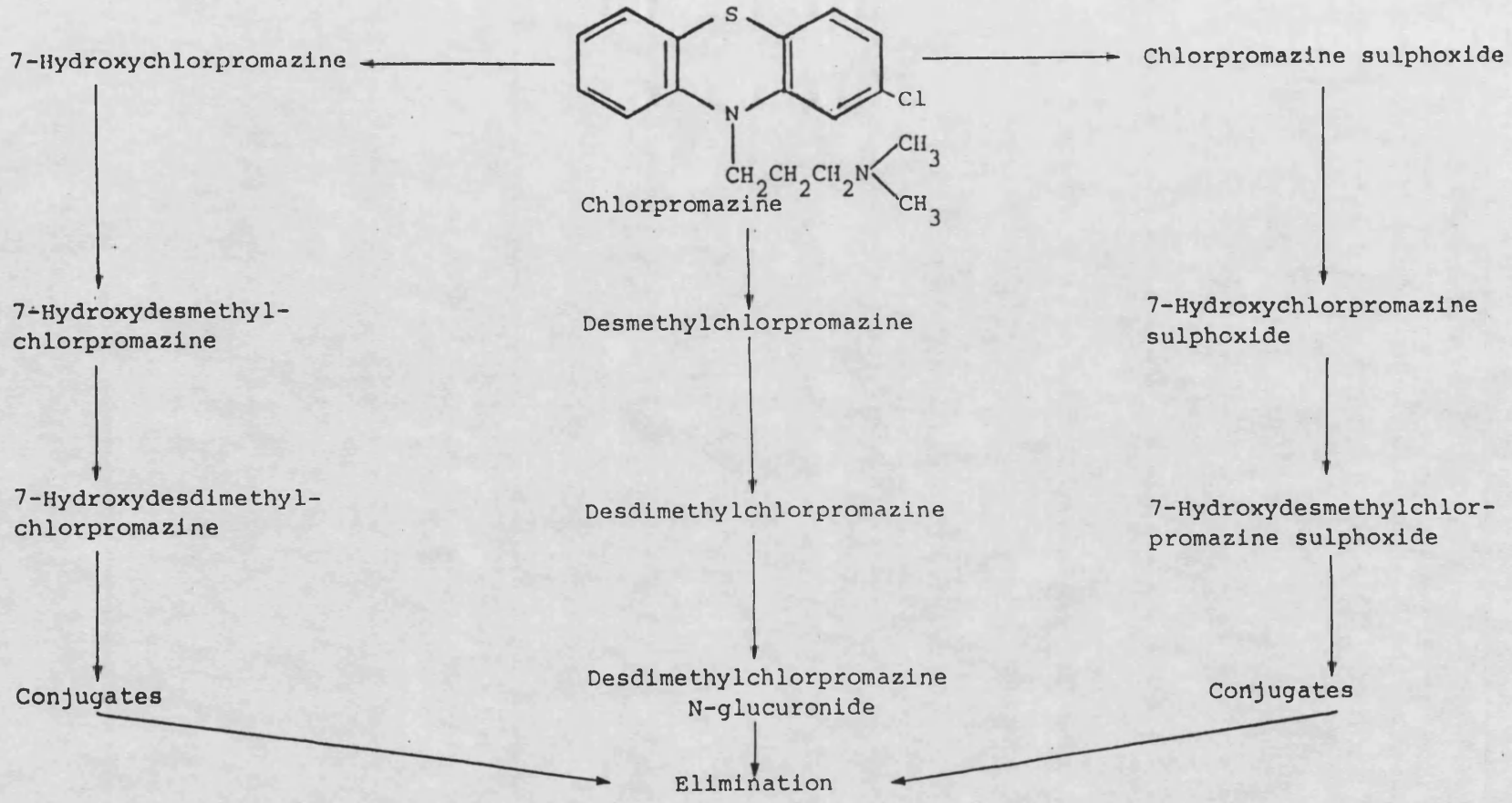


Figure 1·1 Scheme proposed by Wilson et al (1977) for the mammalian metabolism of chlorpromazine

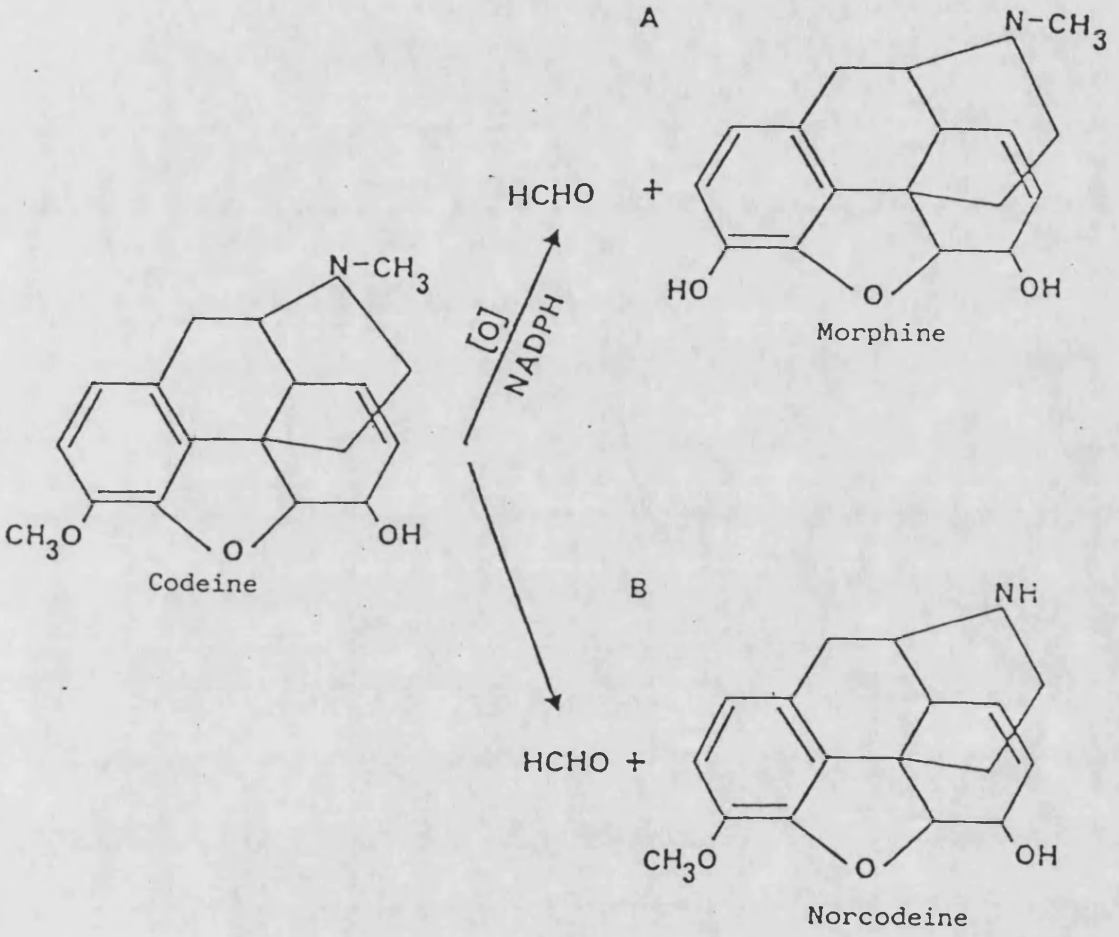
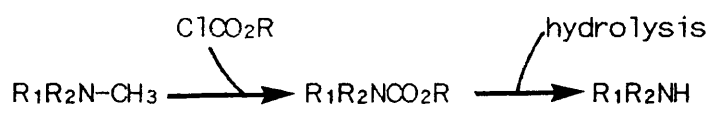


Figure 1·2 Proposed *in vivo* and *in vitro* mammalian metabolism of codeine (Adler and Latham, 1950)

with the cytochrome P-450 monooxygenase is NADPH-cytochrome P-450 reductase which interacts with the P-450 in the terminal electron transport sequence leading to reduction of the cytochrome P-450 (Masters and Okita, 1980). It is not clear how many different forms of inducable liver P-450 exist but it has been suggested that the P-450 systems may be as important in defending the body against foreign chemicals as the immune system is for dealing with foreign pathogens (Marx, 1985).

1.3 Chemical Methods of N-dealkylation

The synthetic chemical method traditionally used for N-dealkylation has been the Von Braun reaction (Hageman, 1953) using cyanogen bromide. This method has usually been used for N-dealkylations of tertiary amines but suffers from the severe disadvantages of very low or variable yields of product, and extreme toxicity of the reagent (Abdel-Monem and Portoghese, 1972). This is especially the case when complex alkaloidal molecules such as codeine are N-dealkylated using the technique (Stenlake, 1979). Attempts have been made to improve on this method by using chloroformate esters. These react with tertiary amines to give carbamates and these are subsequently hydrolysed to give the corresponding amine (Abdel-Monem and Portoghese, 1972):



This reaction sometimes improves selectivity and produces purer products (Palmer and Strauss, 1977). Early studies by Gadamer and Knoch (1921), however, did suggest that the N-methyl function of alkaloids such as morphine and tropine are not cleaved using ethyl chloroformate. Phenylchloroformate and vinylchloroformate are thought to produce more efficient cleavage of tertiary amines than ethyl chloroformate (Olofson *et al*, 1977). The chloroformates, although providing satisfactory yields of N-demethylated products (Rice, 1975) have the disadvantage of being potent lachrymators and hazardous reagents. Other chemical methods of N-demethylation have included using diethylazocarboxylate (Pohland and Sullivan, 1967) and a photochemical method using oxygen (Herlam *et al*, 1973). These methods again suffer from low or variable product yield. These chemical studies suggest a need for alternative N-dealkylation techniques to improve product yield predictions and simultaneously avoid the use of hazardous and toxic reagents.

1.4 Transformations by Microorganisms

1.4.1 History and Scope

The aim of this section is to outline some transformations by microorganisms with some emphasis on pharmaceutically relevant examples.

One of the earliest microbial transformations was reported by

Pasteur in 1864 on the production of acetic acid from ethanol by *Acetobacter aceti*. This was followed by Bertrand in 1896 who reported the formation of l-sorbose from d-sorbitol by the bacterium *Acetobacter xylinum*. Subsequently l-sorbose was used in the chemical synthesis of vitamin C by the Reichstein process (Wells *et al*, 1937). Studies also showed that the use of *Acetobacter suboxydans* produced better yields of l-sorbose and the bacterium is also able to oxidise a number of sugar alcohols (Hann *et al*, 1938).

The first important use of the catalytic capability of microorganisms was due to the finding of Neuberg (1922) that benzaldehyde is transformed to acetylphenylcarbinol by *Saccharomyces cerevisae*. Hildebrant and Klavehn (1934) subsequently patented the production of the sympathomimetic drug ephedrine from the condensation of the acetylphenylcarbinol transformation product with methylamine to produce D(-)-ephedrine. Although ephedrine is now produced almost entirely by chemical synthesis, this example served to demonstrate the potential role of microbial transformations in synthetic processes.

The full potential of microbial transformations in drug synthesis became apparent when Peterson and Murray (1952) synthesised the anti-inflammatory drug cortisone using fungi. The chemical synthesis of cortisone was complex and inefficient requiring large amounts of deoxycholic acid (Sebek and Perlman, 1979). Although an alternative chemical synthesis using progesterone was found, the problems of introducing a hydroxyl group at carbon 11 of the steroid nucleus still remained (McKenzie *et al*, 1948). However using *Rhizopus arrihizus*, progesterone was oxidised to 11

-hydroxyprogesterone (Peterson and Murray, 1952). This method stimulated further studies using microbial transformations to synthesize a variety of corticosteroids (Hubener *et al*, 1951). Careful control of transformation conditions has enabled a range of sterols and steroids to be synthesized (Marsheck, 1971).

Japanese studies in the early sixties first showed that the fungus *Trametes sanguinea* was capable of transforming alkaloids such as thebaine to mixtures of 14-hydroxycodeinone and 14-hydroxycodeine (Tsuda, 1976). In later studies Liras *et al* (1975) reported the bacterial conversion of morphine to 14-hydroxymorphine by an *Arthrobacter sp.*

Most recent studies have described the N-demethylation of codeine by *Streptomyces paucisporogenes*, *Streptomyces lincolnensis* (Sewell *et al*, 1984), *Streptomyces griseus* (Kunz *et al*, 1985) and *Cunninghamella bainieri* (Gibson *et al*, 1984). The transformation of tetrandrine, a potent antitumour drug, to nortetrandrine was also observed with *Streptomyces griseus* (Davis and Rosazza, 1976) and this bacterium is also known to O-demethylate dihydrovindoline (Ecnenrode and Rosazza, 1982).

Antibiotics have also been shown to be susceptible to microbial transformation leading to either complete degradation or limited to hydrolysis, oxidation, reduction, demethylation or acylation (Perlman and Sebek, 1971). For example,the N-demethylation and S-oxidation of clindamycin by *Streptomyces panipalus* and *Streptomyces armentosus* has been described by Argoudelis *et al* (1969). A study was also made of the S-oxidation of lincomycin by *Streptomyces lincolnensis*, the microorganism responsible for lincomycin biosynthesis (Argoudelis and

Mason, 1969). Goldstein *et al* were subsequently able to demonstrate the ability of *Streptomyces erytheus* to O-demethylate the macrolide antibiotic lincomycin.

Although most of the microbial antibiotic transformations have proved to be of little commercial value, the production of 6-amino penicillamic acid (6-APA) from benzyl penicillin using *Escherichia coli* has proved to be commercially important (Perlman and Sebek, 1971). 6-APA has been used as an intermediate in the chemical synthesis of a number of novel penicillin analogues (Vandamme and Voets, 1974).

Microbial transformations have been used to prepare new derivatives of antitumour drugs. The aromatic hydroxylation of ellipticine by *Aspergillus alliaceus* is used to prepare the derivative 8-hydroxyellipticine (Chien and Rosazza, 1979). Another antitumour drug bouvardin is also used for microbial transformation by *Aspergillus ochraceous* and *Streptomyces griseus* to produce O-desmethylbouvardin. This product is used to provide metabolites of bouvardin in quantities sufficient for structure-activity studies (Petroski *et al*, 1978). Kieslich (1976) has surveyed the microbial transformation of non-steroid cyclic compounds and classified hundreds of transformations according to reaction type. These have included oxidation,reduction,hydrolysis,degradation and isomerisation reactions and also the formation of new carbon-carbon or hetero-atom bonds.

It is evident from the above examples of microbial transformations that there has been a proliferation of interest in the chemical actions of microorganisms, and the commercial and

research potential of the microbial P-450 monooxygenases of some of the above reactions is now being realised (Mohr *et al*, 1981).

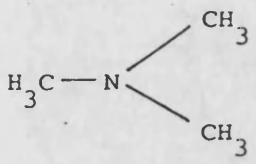
1.4.2 Advantages of Microbial Transformations

Present chemical methods of N-dealkylation necessitate the use of hazardous reagents such as cyanogen bromide and the various chloroformates. Microbial transformations are conducted under mild aqueous conditions and are particularly valuable when reactants or products are susceptable to heat, acids or bases (Wong *et al*, 1979). The enzymes in the microorganisms responsible for the transformations can now to be isolated and current interest is focusing on using the isolated enzymes rather than whole cells in culture. Of particular interest are the cytochrome P-450 monooxygenases, where selective hydroxylation or side chain cleavage reactions of organic compounds on a preparative scale may be pharmaceutically useful (Mohr, 1985). The synthetic chemical hydroxylation of aromatic rings is generally an expensive step in the synthesis of aromatic chemicals. This is often because of the non-specific nature of the hydroxylation reactions where unwanted byproducts are formed leading to inefficient use of starting reagents. The use of microbial P-450 enzymes generally overcomes these disadvantages because of use of aqueous media and reduced use of solvents. At present, the disadvantages of using microbial enzymes are the requirement of expensive product purification procedures,the need to operate under sterile conditions, the high energy consumption of sterilisation procedures and the cost and time involved in isolation of the enzymes (Kieslich, 1980).

1.4.3 N-dealkylations by Bacteria and Fungi

With a view to overcoming the difficulties experienced by medicinal chemists in preparing useful drug intermediates, workers at the University of Bath initiated a study on a microbial transformation system capable of N-dealkylating drug molecules.

The initial studies carried out by Sewell (1982) were concerned with the development of a microbial process with the capacity to N-dealkylate a variety of pharmaceutically important compounds. A series of 'model' compounds were selected for the initial screening experiments with methylotrophic Pseudomonads. This enabled the N-alkyl function to be presented in several of the different chemical enviroments typically encountered in pharmaceutical compounds. The structures of the 'model' test substrates are shown in Figure 1.3. Trimethylamine (1) represented the simplest tertary aliphatic amine. N-methylpiperidine (2) is a simple tertiary amine in a saturated cyclic structure. Codeine (3) is a naturally occuring alkaloid based on a phenanthrene ring structure which is common to the family of morphine-like analgesics. In addition to the N-methyl functions, codeine also possesses a phenolic methyl ether, thus offering sites for both O- and/or N-demethylation. Tropinone (4) represented the molecular nucleus typically found in the naturally occuring tropane family of alkaloids. Pentazocine (5) is a typical example of a 6,7-benzomorphan analgesic. This was included in the screening programme to determine the ability of the test microorganisms to cleave a bulky nitrogen substituent.



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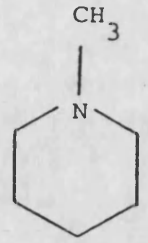

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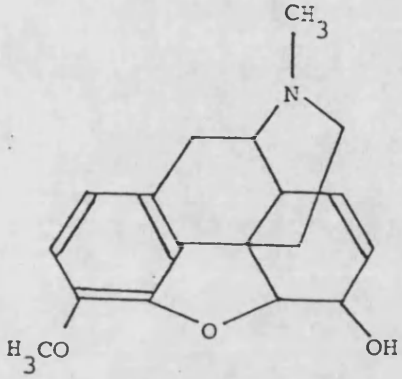

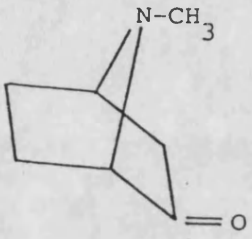

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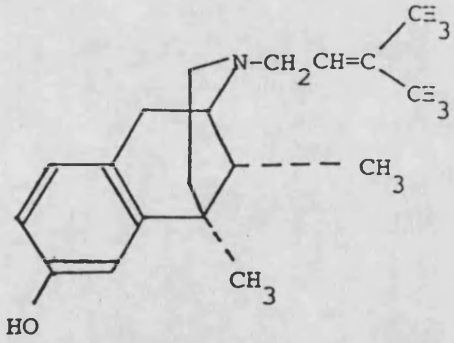

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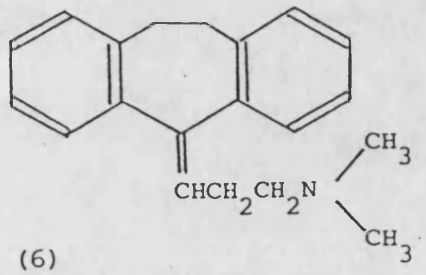
Figure 1·3 Test substrates used for screening experiments with methylotrophic Pseudomonads by Sewell (1982)

The screening experiments with methylotrophic Pseudomonads suggested that the N-dealkylating capability of these microorganisms was limited to simple aliphatic amines, such as trimethylamine. The screening programme was subsequently extended to include strains of *Streptomyces* and fungi, mainly species of *Cunninghamella*. In these studies 'model' compounds were again selected to be representative of the main classes of nitrogenous drug molecules of pharmaceutical interest (Figure 1.4).

Amitryptyline (6) a tricyclic antidepressant drug with a dimethylamino group may be N-demethylated to produce nortryptiline, and again to produce desmethylnortryptyline. Chlorpromazine (7), a central nervous system depressant, may be N-demethylated at the dimethylaminopropyl side chain to give the secondary amine (8) desmethylchlorpromazine and again to produce the desdimethyl derivative. In addition the entire dimethylaminopropyl group may be cleaved from the phenothiazine atom. Desmethylchlorpromazine (8) was therefore screened separately as it presented a secondary amine system for transformation. Diazepam (9) is a member of the benzodiazepine group of minor tranquilizers, and was included because it is a cyclic amine and not an amide. Metazocine (10) is a synthetic analgesic representitive of the 6,7-benzomorphans. Codeine (3) was also screened to test the selectivity of microorganisms for O and/or N-demethylation. These microbial transformation experiments were conducted in liquid culture, and demonstrated the ability of several of these microorganisms to N-demethylate the drug molecules.

A few of the *Streptomyces* species were able to transform one or two of the test compounds; *Streptomyces lincolnensis* N-demethylated

Figure 1·4 Chemical structures of substrate compounds selected for screening with Streptomyces sp. and fungi by Sewell (1982).



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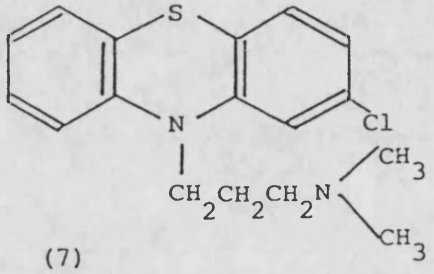

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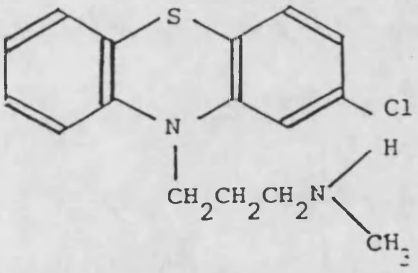

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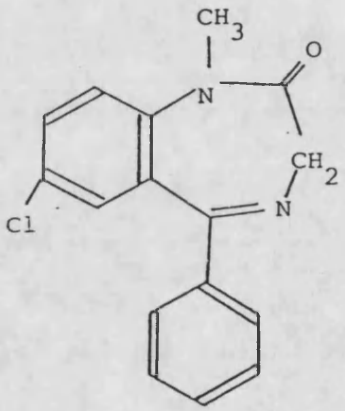

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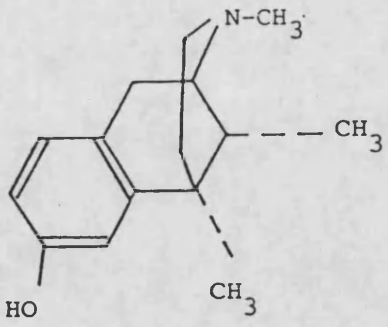


(10)

codeine,metazocine and amitriptyline. *Streptomyces rimosus* N-demethylated diazepam and amitriptyline and *Streptomyces paucisporogenes* N-demethylated codeine only. The most promising results were obtained with the screening of *Cunninghamella* sp. where all the test substrates,with the exception of pentazocine,were N-dealkylated by several species of this genus (Sewell, 1982).

On continuing these studies, Sewell (1982) developed defined and semi-defined growth media to facilitate the quantitative investigation of various growth medium parameters on N-demethylation. *Cunninghamella echinulata* IMI 199844, was selected as the test organism since the strain had demonstrated the ability to N-demethylate a wide range of compounds with product yields greater than those achieved by other organisms. Codeine was selected as the test substrate for these studies. In order to quantify the transformation results, norcodeine the transformation product, was estimated by gas chromatography assay and the microbial growth was determined by dry cell weight. On the basis of both microbial growth and codeine transformation, Sewell (1982) was able to ascertain the most suitable growth medium for further transformations. Low conversions from codeine to norcodeine were obtained for experiments conducted in culture media containing carbon sources eg. maltose which supported a rapid microbial growth rate. Cultures containing a less utilised carbon source such as succinate produced higher transformation yields of norcodeine. These results led Sewell (1982) to speculate on a carbon source regulatory mechanism.

Further studies with *Cunninghamella echinulata* disclosed that it exhibited typical pelleted fungal growth with cube root rather than

exponential kinetics. The growth curve, of dry cell weight against incubation time exhibited a short lag period, followed by a slow growth phase which continued up until the fourth day of incubation. Cell lysis, measured as the leakage of 260nm absorbing substances from the cell, and extracellular protein concentration, both increased until the seventh day of incubation where a plateau was reached. This suggested that codeine transformation was not dependant upon cell lysis as a means of enabling contact between the codeine substrate and the N-demethylating enzyme system. The extent of fungal growth was found to be limited by carbon source exhaustion which occured between the fifth and sixth days of incubation. Codeine N-demethylation occured only after the glucose carbon source had been depleted, providing further evidence for the presence of a carbon source regulatory mechanism. This was attributed to catabolic repression rather than catabolic inhibition,supported by subsequent experiments with resting cell suspensions.

Having demonstrated the ability of *Cunninghamella echinulata* IMI 199844 to N-demethylate a variety of different substrates in the screening programme, Sewell (1982) proceeded to attempt to characterise the enzyme responsible for N-demethylation. To do this it was necessary to extract the N-demethylase from the fungal cells in order to study the enzyme, independent of enzyme production and cell permeability effects.

A cell-free extract was prepared from *Cunninghamella echinulata* which retained a measureable degree of N-demethylase activity. The success of various enzyme extraction procedures was measured in terms of the supernatant protein concentration after the removal of cell

debris. Sewell (1982) found the most effective extraction procedure was homogenisation, with subsequent treatment of the homogenate with Triton X-100, a nonionic surfactant. The homogenisation process was observed to disrupt the mycelial pellets, and the Triton X-100 was thought to solubilize the hydrophobic part of the enzyme with the cellular membrane fragments. The active component of the cell-free extract behaved as a typical monooxygenase enzyme since codeine N-demethylation was inhibited by anoxic conditions, carbon monoxide and SKF 525A. Carbon monoxide and SKF 525A are classical inhibitors of monooxygenases of both mammalian and microbial origin (Kanematsu *et al*, 1969). Sewell (1982) therefore suggested that the *Cunninghamella echinulata* codeine N-demethylase may be a typical cytochrome P-450 linked monooxygenase.

Follow up work by Gibson (1984) using *Cunninghamella bainieri* also provided strong evidence that the N-demethylase enzyme was a cytochrome P-450 monooxygenase. Data from the kinetic studies of codeine transformation by Gibson (1984) implied that codeine N-oxide was not an intermediate in the N-demethylation of codeine by *Cunninghamella bainieri*. Using selective enzyme inducers and inhibitors, Gibson proposed that codeine N-demethylation occured via x-C oxidation rather than N-oxidation to produce a carbinolamine intermediate (N-hydroxymethylcodeine) (Figure 1.5). A ^{13}C spectroscopic technique provided further evidence for the proposed mechanism.

Due to the growth characteristics and filamentous nature of *Cunninghamella* sp. problems were encountered when scale up studies

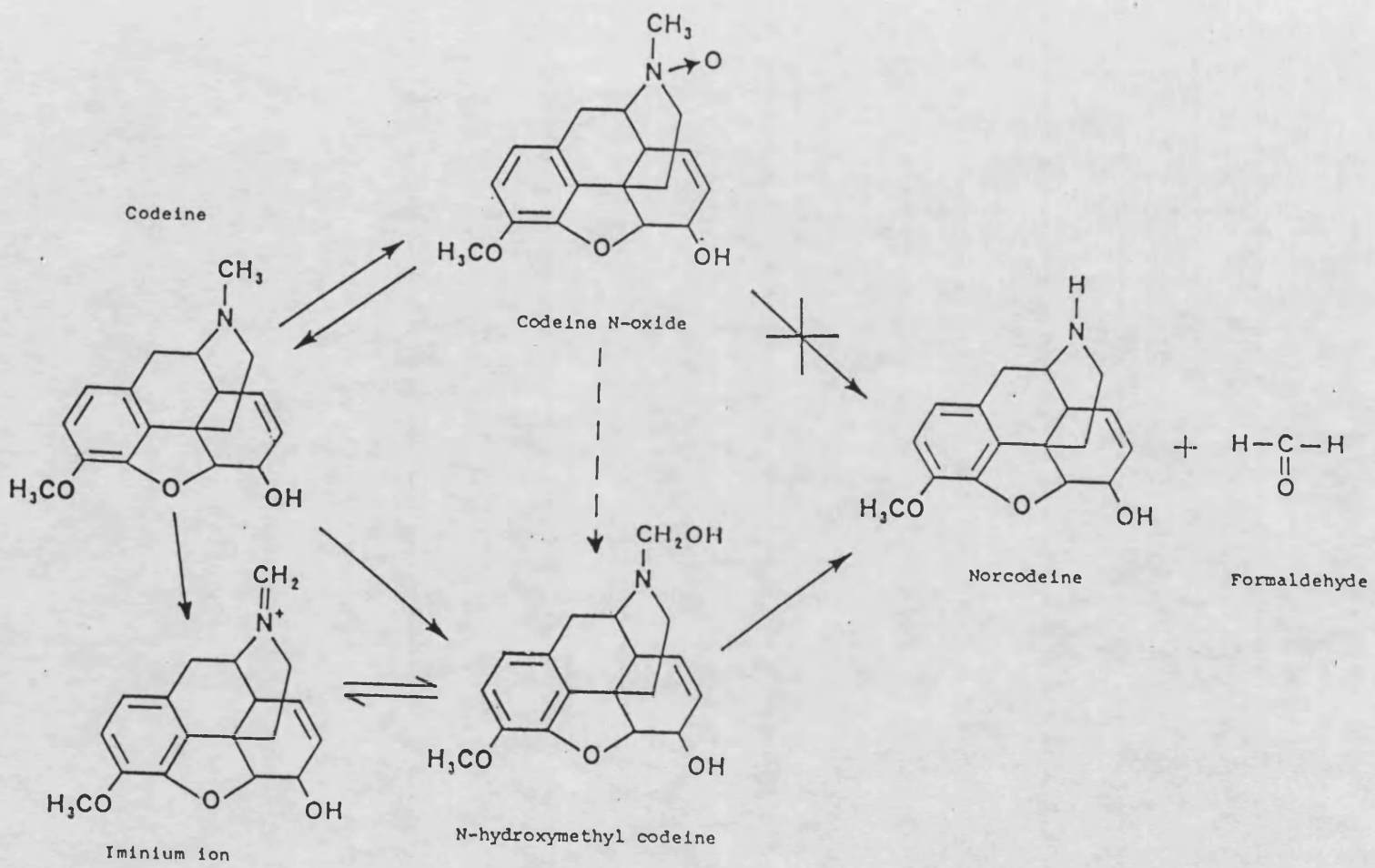


Figure 1·5 Mechanism proposed by Gibson (1984) for N-demethylation of codeine by cell-free extracts of Cunninghamella bainieri.

were attempted in the fermenter (Gibson, 1984). These included localised growth around air vents and propellers thereby preventing homogenous growth in the medium. *Cunninghamella bainieri* also required up to 10 days incubation for N-demethylation to be achieved. It was therefore decided to find other N-demethylating organisms, preferably unicellular to overcome these problems. The studies by Gibson (1984) suggested that a cytochrome P-450 type demethylase enzyme was responsible for the N-demethylations by *Cunninghamella* sp. and there was strong evidence for the presence of cytochrome P-450 enzymes in *Candida* sp. (Lebeault *et al*, 1971). *Candida* was therefore chosen as a suitable unicellular organism for further investigations in the present work.

1.5 The genus *Candida*

1.5.1 Taxonomic Classification

Class: Fungi imperfecti

Order: Moniliales

Family: *Cryptococcaceae*

155 species of *Candida* have been described (Barnett *et al*, 1983).

1.5.2 Characteristics of the genus

Examples of the genus show well marked diamorphism. *Candida* sp.

exist as budding yeasts, particularly where the concentration of simple carbohydrates is high and the culture is well aerated or they exist as pseudo or septate mycelia producing blastospores (Robinson, 1978). With glucose as the carbon source, only yeast-like cells are formed, but in media containing soluble starch and low nitrogen concentration, pseudomycelia predominate.

Candida sp. grow best in liquid culture at pH 6 - 6.5 and temperature 28°C, although pathogenic strains eg. *Candida albicans* are able to grow at temperatures between 28°C and 37°C. The microscopic appearance of the type species *Candida tropicalis* is of ovoid yeast like cells 3-9μ by 4-12μ with multilateral budding and no filaments or hyphae. The cell wall consists of two layers and the characteristic monosaccharide components are glucose,mannose and glucosamine.

Candida sp. do not undergo sexual reproduction. Asexual reproduction is by budding where a daughter cell develops from a small outgrowth of the parent cell. This requires the synthesis of additional cell wall material and appears to involve the coordinated activities of autolytic and synthetic enzymes. During the budding process,mitotic division occurs in the parent cell and one of the post division nuclei passes into the daughter cell. Since *Candida* is a multipolar yeast, the daughter cells may arise at any of a number of sites on the parent cell.

1.5.3 Pathogenicity of *Candida* sp.

Candida sp. have been isolated from healthy human skin and are

the most commonly occuring skin yeasts (Warnock and Richardson, 1982). The pathogenic species *Candida albicans* can also grow saprotrophically in man. It requires an adequate oxygen supply and the buccal cavity would appear to provide the most favorable conditions. *Candida albicans* is also found in the urogenital system and the vagina.

The infection caused by *Candida* sp. is called candidosis candidiasis or moniliasis and can be superficial or systemic (Warnock & Richardson, 1982). The usual sites of superficial infection are skin folds, the anal,vulvovaginal and perioral mucocutaneous junctions and the nail bed and nails. Systemic candidosis is characterised by mycelial invasion of tissues or the formation of intravascular thrombi and granulata. The lesions can occur at a number of sites including the alimentary tract, the heart the urogenital tract,the repiratory tract, the heart,bones and the central nervous system.

Eight species of *Candida* have been found to be pathogenic for man: *C. albicans, C. stellatoidea, C. tropicalis, C. parapoilosis, C. krusei, C. pseudotropicalis, C. guilliermondii* and *C. glabrata* (Warnock & Richardson, 1982).

1.5.4. Biotransformations by *Candida* sp.

Candidia sp are able to ferment monosaccharides such as glucose, maltose or trehalose, but are usually unable to ferment lactose and other disaccharides. The exceptions are *Candida tropicalis* which can ferment sucrose and *Candida pseudotropicalis* which produces a

B-galactosidase and can also ferment lactose. The carbohydrates are metabolised to the corresponding polyalcohols.

Candida sp also have the capacity to transform higher aliphatic hydrocarbons (Lebeault *et al* 1971). Three mechanims have been reported for the initial attack on n-alkanes:

i) Hydroxylation by mixed function monooxygenase systems

ii) Dehydorgenation to corresponding alkenes

iii) Hydroperoxidation (Duppel *et al* 1973).

In hydroxylation by mixed function monooxygenases, molecular oxygen is incorporated into n-alkanes to give the corresponding primary alcohol and the monocarboxylic acid of the same chain length as the substrate. The oxidation of long chain alcohols to corresponding fatty acids by *Candida albicans* is diterminal, leading to a dicarboxylic acid. With *Candida tropicalis* both mono- and diterminal oxidation have been observed (Lebeault *et al* 1971). The metabolism of fatty acids via B-oxidation has been observed in *Candida lipolytica* (Lebeault *et al* 1971). In this pathway the synthesis of acyl-CoA is the key step. This is converted to acetyl CoA which enters the glyoxylate cycle. In this cycle the production of one C_4 compound occurs as malate or succinate. The β-oxidation of fatty acids to corresponding β-hydroxy derivatives has also been observed in *Candida lipolytica* (Lebeault *et al* 1971). The observation that *Candida* sp were able to transform hydrocarbons has encouraged the investigation of their potential as metabolisers of other organic compounds. *Candida krusei, Candida utilis, Candida pulcherina and Candida guiliermondii* have been reported to transform sarsasapogenin, diosgenin and 4-dehydrotigoggenone to a variety of oxidised and

demethylated compounds (Gallo *et al*, 1971). Recent investigations by Hitchcock *et al* (1989) have concerned the ability of *Candida albicans* to demethylate lanosterol. The demethylation of lanosterol has been demonstrated to proceed via a specific cytochrome P-450 enzyme (Hitchcock *et al* 1989) where the 8 double bond of lanosterol plays an important role in the interaction of cytochrome P-450 and substrate (Aoyama *et al* 1989).

1.6. Cytochrome P-450 of Yeasts

1.6.1. Physiological Role

Whilst the biotransformations using fungi were being studied, Lindenmayer and Smith in 1964 first produced cellular extracts containing cytochrome P-450 from the yeast *Saccharomyces cerevisiae*. Subsequently, yeast P-450 studies have been focusing on the hydrocarbon degrading yeasts of the genus *Candida*. It was postulated that P-450 was the enzyme involved in the terminal hydroxylation of n-alkanes and this physiological role of the n-alkane induced P-450 in *Candida sp*. has been confirmed (Sanglard *et al*, 1984). The biological role of P-450 in *S. cerevisiae* remained unclear for a long time until Ohba *et al* (1978) confirmed its involvement in the late stages of ergosterol biosynthesis in the yeast cell wall. It is now accepted that the non induced cytochrome P-450s in yeasts effect the removal of the 14δ methyl group of lanosterol during ergosterol biosynthesis (Aoyama *et al*, 1981a) (Figure 1.6). However, Hata *et al*

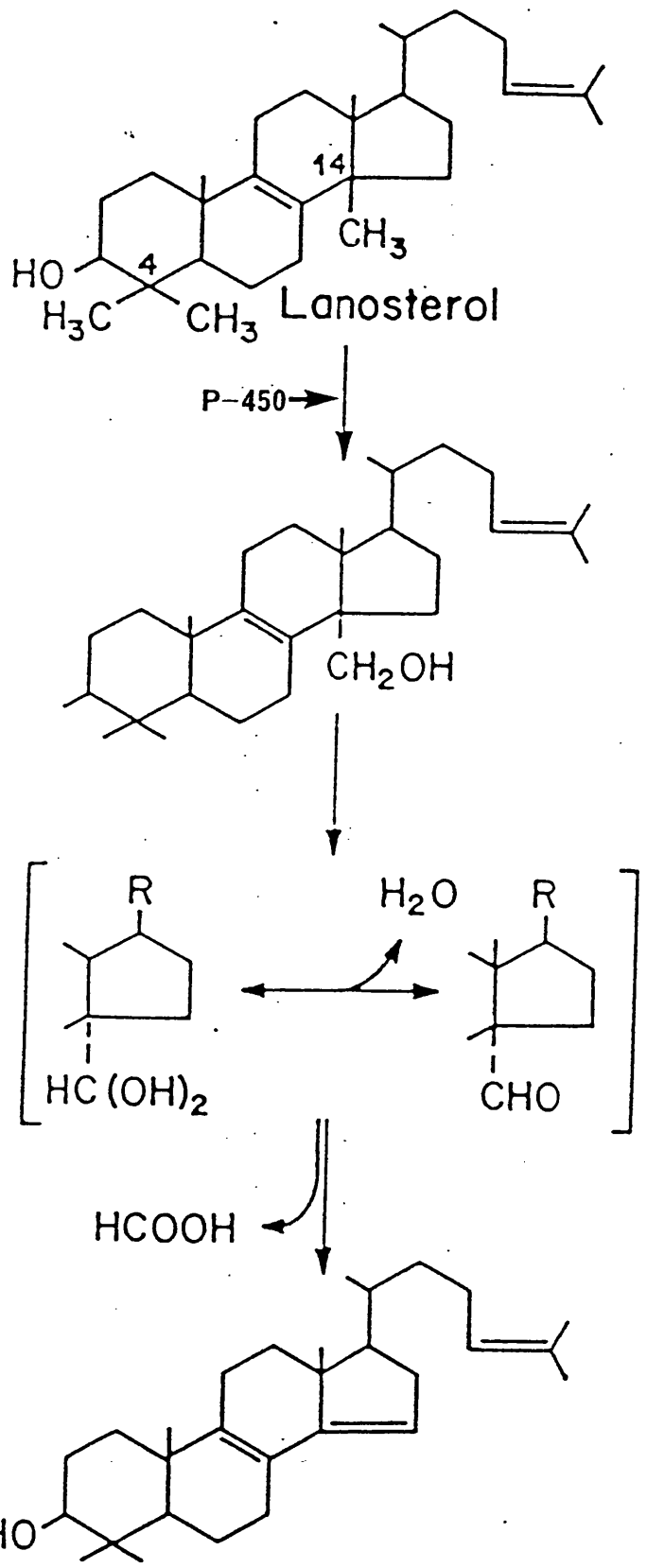


Figure 1·6 Proposed scheme for the demethylation of lanosterol by cytochrome P-450 (Aoyama et al , 1981a)

(1982) have shown that the P-450 of yeast strains lacking 14δ demethylation activity are able to effect the $^{22}\Delta$desaturation of ergosta-5,7-diene-3B-ol, another sterol intermediate in ergosterol biosynthesis. This suggests that two distinct species of cytochrome P-450 may be involved in ergosterol biosynthesis. Besides the important involvement of P-450s in lipid biosynthesis, another possible physiological role of the enzyme has been suggested; mitochondrial respiration usually creates the yeast's energy requirement when sufficient oxygen is available, but when oxygen becomes limited another pathway involving cytochrome P-450 may become effective where NADH and NADPH are regenerated (Ainsworth *et al*, 1980).

1.6.2. Distribution and Induction of Cytochrome P-450 in Yeasts

In hydrocarbon utilising yeasts such as *Candida sp* the P-450 is thought to be induced by the substrate. The P-450 content of cells is also linearly related to substrate uptake rate (Gmunder *et al*, 1981a). Very low oxygen availability together with n-alkane substrates act as inducers of P-450 production but glucose is found to act as a repressor (Mauersberger *et al*, 1980). Studies have shown that partially purified preparations from alkane grown *Candida sp* are able to N-demethylate aminopyrine, benzamphetamine, ethylmorphine and various other drugs (Lebeault *et al*, 1971; Duppel *et al*, 1973).

The formation of cytochrome P-450 in *Saccharomyces cerevisiae* is dependant entirely on different factors than in alkane assimilating yeasts. Studies have suggested that high glucose concentrations and

low rates of respiration by decreased oxygen levels are the conditions needed for P-450 induction (Wiseman and King, 1982). The catalytic activity of the P-450 from *S. cerevisiae* also differs markedly under different conditions, where 14δ demethylation of lanosterol is the characteristic activity which has been verified by studies by Aoyama (Aoyama *et al*, 1983; 1984). Subsequent data has indicated that the P-450 of glucose grown *Candida tropicalis* very much resembles that of *Saccharomyces cerevisiae* regarding N-demethylation activity (Kappeli *et al*, 1985). So far the ability of a yeast to form two distinct types of P-450 depending on substrate source, has only been found with the *Candida tropicalis* strains.

Karenlampi *et al* (1982) have obtained data on the distribution of cytochrome P-450s in various other yeasts and found measurable levels in *Brettanomyces anomalus, Hansanula anomula, Saccharomyces bayanus* and *Saccharomyces italicus*. These studies also showed that only two out of fourteen chemicals known to be hepatic P-450 inducers, increased the P-450 content in yeasts, where an average 30% increase was observed. Wiseman and Lim (1975) obtained similar results with yeasts grown on phenobarbital a known inducer of mammalian P-450. However, these studies only measured the P-450 content and not P-450 activity.

1.6.3 Properties of Yeast Cytochrome P-450s

The molecular weight of the monomeric form of P-450 from *Saccharomyces cerevisiae* has been determined to be 55,500 (King *et al*, 1984) and 58,000 (Yoshida and Aoyama, 1984) and these values are

similar to those determined for mammalian P-450 (Yoshida and Aoyama, 1984). The most distinctive spectral property of yeast P-450s is their absorbance at around 450 nm in the reduced CO difference spectrum and this spectral property is common with the mammalian P-450s. Like hepatic P-450s, yeast P-450s can be denatured to cytochrome P-420. Denaturing agents include several organic solvents, high detergent concentrations and mercurials such as p-chloromercuribenzoate (Yoshida and Aoyama, 1984). However, yeast P-450s are considered more resistant to non-ionic detergents than hepatic P-450s (Riege *et al*,1981).

1.7 The Cytochrome P-450 System

It was Klingenberg (1958) and Garfinkel (1958) who independently announced the discovery of a CO binding pigment (haemoprotein) in the microsomal fraction of rat hepatic liver. It was later Omura and Sato (1964) who defined the new haemoprotein as a "b type" cytochrome and called it cytochrome P-450 from the peak absorbance of its CO complex. At present the full mechanism of P-450 catalysed reactions is still not fully understood. Much of the insight gained in the past 20 years into the structure, mechanisms and spectra of the enzyme has been based on work with cytochrome P-450 camphor (P-450 cam) from *Pseudomonas putida*, which is soluble. This is in contrast to yeast and mammalian P-450s which are mostly membrane bound. The aim of this section is to summarise the present understanding of the cytochrome P-450 system.

All haemoproteins have a common active site or prosthetic group

consisting of an iron porphyrin (haem) complex which is almost an independent planar entity embedded in the globular protein. The haem unit is attached to the remainder of the protein by one or two covalent bonds to nearby amino acid residues which function as axial ligands. The biological function of all classes of haem proteins is centred on the haem group and on the iron itself. Therefore the oxidation state of the iron, nature of axial ligands and the protein environment of the haem unit, serve as subtle modulators of biological behaviour. The haem group is also the main origin of the spectral features of these proteins.

In cytochrome P-450, the ferrous haem-carbonyl absorption maximum near 450nm is different from that exhibited by typical haem proteins such as myoglobin (435nm) and horseradish peroxidase (438nm). Mason *et al* (1965) were the first to suggest that the unusual spectral properties of cytochrome P-450 are attributable to the presence of a thiolate sulphur atom ligated to the haem iron atom in the fifth or proximal coordination site. the most recent proof of this suggestion has come from X-ray crystallographic studies on bacterial cytochrome P-450$_{cam}$ (Poulos *et al*, 1988). In the ferric state, cytochrome P-450s exist in either a low-spin hexacoordinate (b) form or a high-spin pentacoordinate form (c), where the low spin type is more predominant (Black & Coon, 1986) (Figure 1.7).

The protein portion is characterised by its primary structure (the amino acid sequence of the polypeptide chain) and its stereo structure (the arrangement of the amino acid residues). The electronic configuration of the iron porphyrin is responsible for the functional specificity of the haemoproteins. The electronic

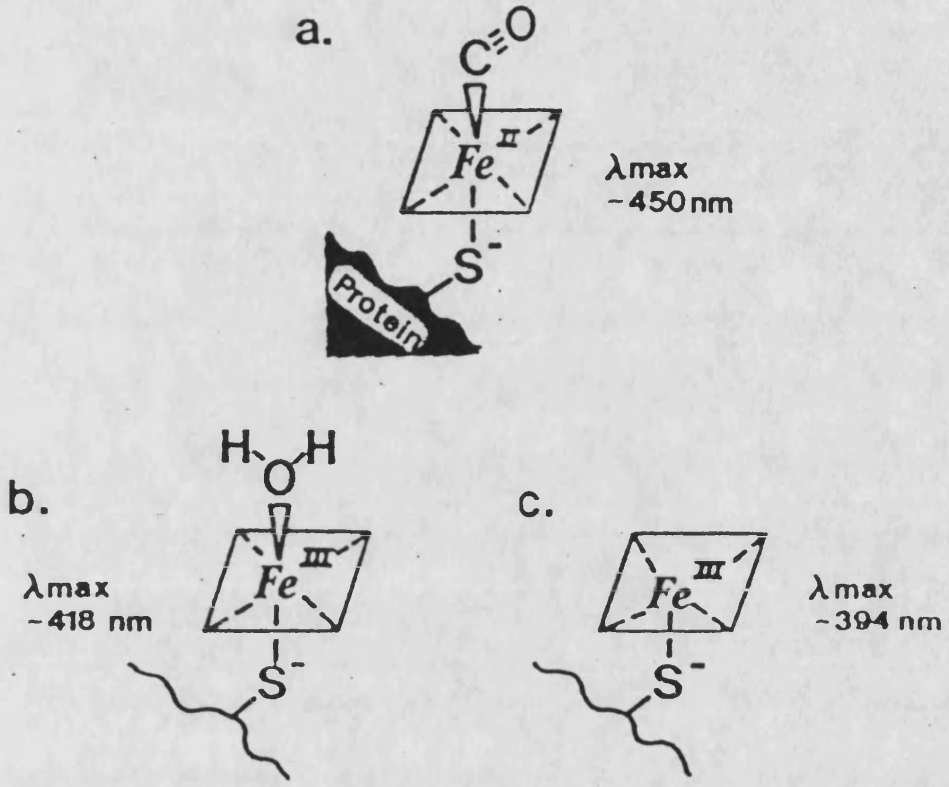


Figure 1·7 Schematic representation of active site structures of cytochrome P-450 with the (a) ferrous carbonyl state, (b) low spin ferric state and (c) high spin ferric state.

configuration depends largely on the charge of the iron (ferric or ferrous) and on the protein-porphyrin interactions (cis and trans effects). Therefore the protein greatly influences the behaviour of the haemoprotein and it is this that differentiates the haemoproteins from simple iron porphyrin complexes such as myoglobin.

Two classes of cytochrome P-450 monooxygenases have been identified. The two classes are defined by the character of the electron transport chain (Figure 1.8) (Murray*et al* 1985). The bacterial and mitochondrial systems (a) have three protein components,utilise NAD(P)H as the electron donor and contain an FAD-flavoprotein reductase which receives two electrons from NADPH. Single electrons are then passed via the iron sulphur centre ($Fe_2S_2Cys_4$) to the P-450 haem active centre. In microsomal monooxygenases (b),present in yeast and liver microsomes (Gunsalus & Sligar, 1976), NADPH provides two electrons to the FAD prosthetic group of a more complicated FAD/FMN-flavoprotein reductase. The electrons are then passed to the haem of the cytochrome P-450 (White & Coon, 1980).

Apart from substrate monooxygenation (hydroxylation),numerous other chemical transformations involving cytochrome P-450 have been reported including; epoxidation, peroxygenation,N-,S-, and O-dealkylation,N- and S-oxygenation, desulphurisation, deamination, isomerisation and nonhydrolytic carbon-carbon bond cleavage (Black & Coon, 1986).

Many of the liver microsomal P-450s exhibit nonselectivity and are individually capable of binding and effecting the oxygenation of thousands of different compounds at different rates. The substrates

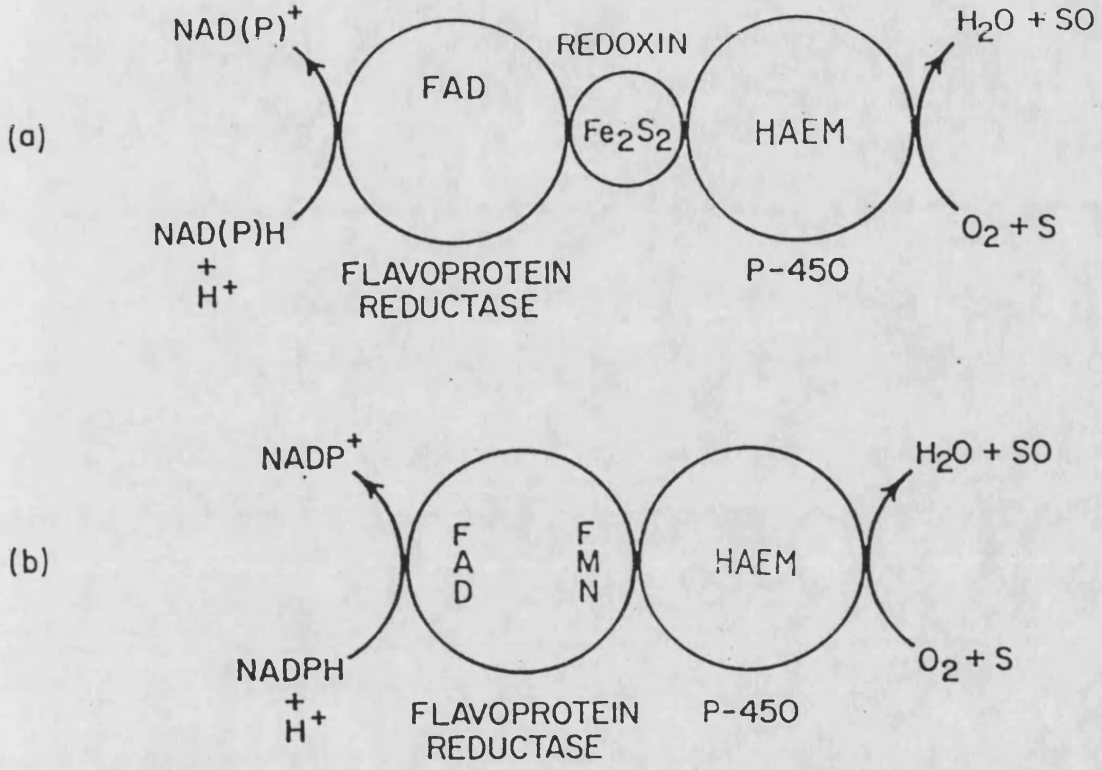


Figure 1·8 Schematic diagram of (a) bacterial/mitochondrial and (b) microsomal cytochrome P-450 electron transfer chains

are mostly hydrophobic in character and the extent of binding to the cytochrome P-450 is determined largely by the degree of non-polarity (White & Coon, 1980). The microsomal cytochrome P-450s show broad and overlapping specificity and are believed to be the most non-specific enzymes known (Coon & Koop, 1983). In contrast, the mitochondrial and bacterial P-450s are generally are substrate specific. They act only on a few substrates and exhibit high specificity for the position attacked and the stereochemistry of the reaction (Gunsalus & Sligar, 1978).

The cytochrome P-450s act on both physiologically occurring lipids and on foreign compounds. With the latter, thousands of different foreign drug molecules are oxidised to more water soluble and generally less pharmacologically potent derivatives. These are then excreted either directly or after conjugation of the products with glucuronic acid or glutathione (Williams, 1959). The phenomenon of drug tolerance is, in many instances, due to the induction of drug metabolising forms of cytochrome P-450. The resulting increase in oxidative inactivation of a given drug then results in a requirement for higher doses to achieve the desired therapeutic effect. Other xenobiotics that are effectively metabolised and detoxified by the cytochrome P-450 monooxygenase system include halocarbons such as carbon tetrachloride, halothane,polybrominated biphenyls and commonly ingested plant or mould metabolites such as aflatoxins, caffeine, theophylline and isosafrole (Guengerich & Liebler, 1985). However, xenobiotics are not always detoxified by the action of cytochrome P-450s. For example, the relatively harmless aryl hydrocarbon benzo(a)pyrene, which is readly formed in charred or burned organic

material, is converted to a highly mutagenic and carcinogenic diol-epoxide derivative by the action of cytochrome P-450 and epoxide hydroxylase (Deutsch *et al*, 1978).

A schematic representation of the present concept of the mechanism of P-450 catalysed reactions is shown in Figure 1.9. The reaction starts with the binding of the substrate R-N to the terminal oxidase. This binding alters the spin state of the iron depending on the structure of the substrate (Rein and Ristram, 1978). This is followed by reduction of the P-450 Fe^{3+}-protoporphyrin to Fe^{2+} by electron transport from NADPH to the iron by cytochrome P-450 NADPH reductase. This first reduction is thought to be controlled by the P-450 protein bound to the substrate (Matsubara *et al*, 1976). In the next step the ferrous P-450-substrate-oxygen complex is formed by binding of molecular oxygen and this is followed by insertion of an electron by the P-450 reductase. In the final step hydroxylation of the bound substrate occurs. This final step may be broken down into various steps which result in an "active" oxene complex $(Fe-O)^{3+}$, which is thought to be responsible for the transformation of oxygen into the substrate (Groves *et al*, 1978). The exact mechanism for this transport is still unknown.

In the absence of cytochrome P-450 the activation energy for the overall reaction is about 0.42J/mol, however in the presence of enzyme the activation energy is decreased to about 0.042J/mol. Therefore the cytochrome P-450 induces a decreases in the dissociation enthalpy of oxygen (Hamilton and Hayaishi, 1974). The rate limiting steps in the overall reaction cycle may vary and Lu *et al* (1984) have suggested multiple rate limiting steps may exist in

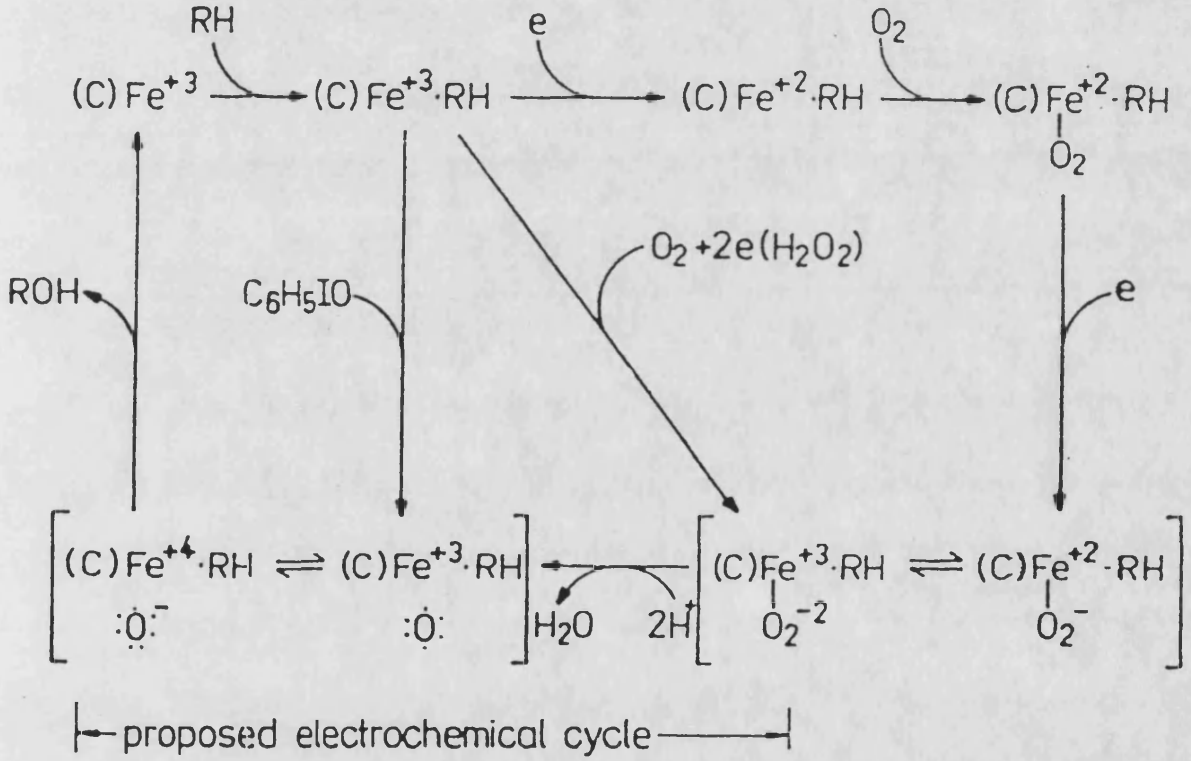


Figure 1·9 Schematic representation of the present concept of the mechanism of cytochrome P-450 catalysed hydroxylation reactions.

P-450 catalysed reactions.

Current research into the structure and function of cytochrome P-450s may be helped by the emergence of the azole antifungal drugs which are effective inhibitors of yeast cytochrome P-450. The inhibition of yeast P-450s is known to be the primary mode of action of these drugs and they are now beginning to be used to study the binding of substrate/inhibitor to cytochrome P-450. It has been shown that both binding and inhibition are dependant on the presence of a sterically unhindered N atom of an azole ring eg imidazole or triazole (Wilkinson *et al*, 1972). A hypothetical model of the interaction of the drug itraconazole with cytochrome P-450 has now been presented (Tollenaere and Janseen, 1988) and shown in Figure 1.10. Figure 1.10 shows the N-atom at position 4 of the triazole ring binds to the heam iron and the rest of the molecule binds to the protein near the active site. These studies may therefore provide a route for the parallel advancement for both the search for novel fungicidal drugs and a greater understanding of cytochrome P-450 enzymes.

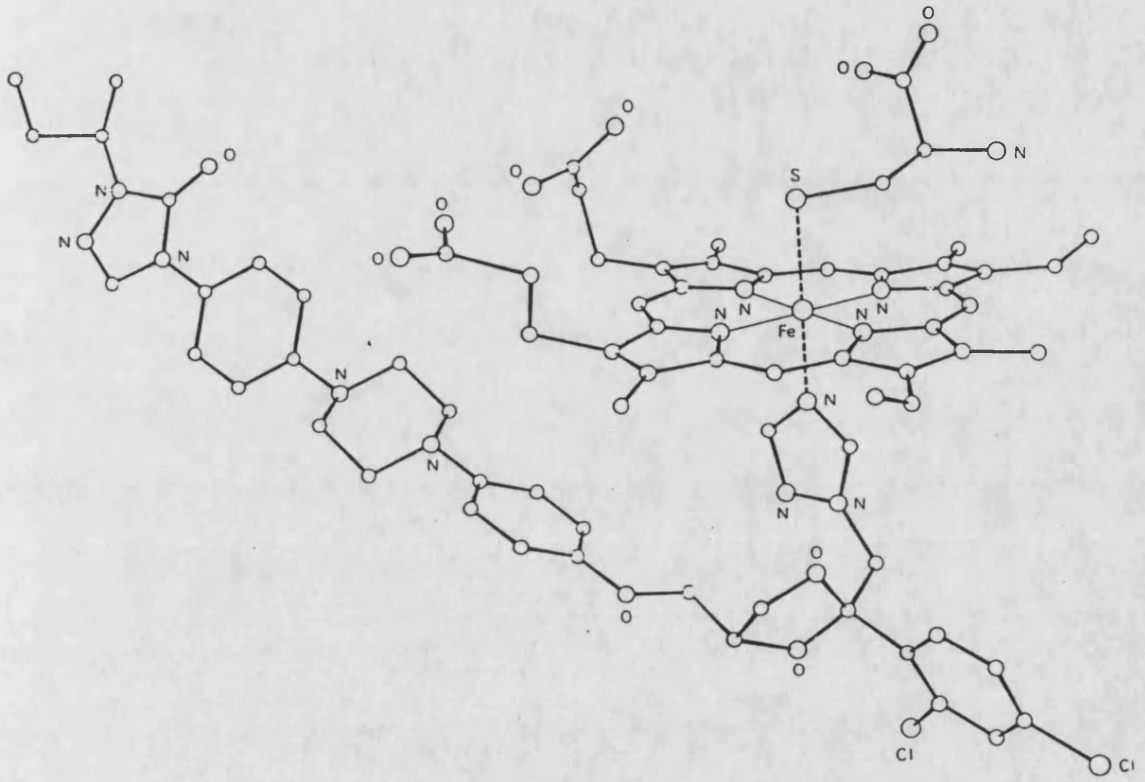


Figure 1·10 Hypothetical model of the interaction of the imidazole antifungal drug itraconazole with cytochrome P-450

CHAPTER TWO

SCREENING OF *Candida tropicalis* STRAINS FOR N-DEMETHYLATION ACTIVITY

CHAPTER 2. SCREENING OF *Candida tropicalis* STRAINS FOR N-DEMETHYLATION ACTIVITY

2.1 Introduction

Prior to identifying the difficulties of direct chemical methods of N-dealkylation, studies in Japan had shown the ability of fungi such as *Trametes sanguinea* to oxidise thebaine to 14-hydroxycodeine (Iizuka *et al*, 1960). Later work by Liras *et al* (1975) also showed the ability of bacteria such as *Arthrobacter sp.* to transform morphine to 14-hydroxymorphine and this activity was also shown with *Pseudomonas testosteroni* (Liras *et al*, 1975). Since then studies have been done on the ability of various bacteria and fungi to N-dealkylate various drug molecules including codeine, and these studies were an attempt to provide an effective alternative to chemical methods of N-dealkylation (Sewell, 1982; Gibson, 1984). The opiate alkaloid codeine was chosen as a "model substrate" because of its capacity to be both N- and O- dealkylated. Sewell (1982) showed that strains of *Cunninghamella sp.* were able to N-demethylate codeine in satisfactory yields. Gibson (1984) carried out further detailed studies on the biochemistry of codeine N-demethylation. These studies, however necessitated the use of fermenters to scale up growth of the fungi but major problems were encountered because of surface attachment of fungal growth to fermenter parts.

Clearly an alternative microorganism was needed which would easily grow in the fermenter whilst having N-dealkylation capacity.

The *Candida sp* have been characterised regarding their ability to transform various organic chemicals (see Section 1.5.4.) and they have the advantage of growing as single non-filamentous cells enabling easy fermenter cultivation (Duppel *et al*, 1973; Gmunder *et al*, 1981a). Furthermore, their doubling time in culture is also considerably shorter than that of *Cunninghamella sp*. Several strains of *Candida* have been investigated recently for N-demethylation activity using codeine as a test substrate (Stavrianakis, 1986). These studies showed that *Candida tropicalis* in particular was able to N-demethylate codeine but that the activity was poor probably because of the growth medium and uncontrolled cultivation conditions used. Strains of *Candida tropicalis* therefore have been chosen in this work for further detailed studies. Species selection of *Candida tropicalis* was also based on their reported ability to carry out transformations of chemicals (Lebeault *et al*, 1971), the demonstrated inducibility of their transformation enzymes (Duppel *et al*, 1973) and their ease of growth in fermenters.

Microbial transformation studies have traditionally been carried out in liquid cultures and this method was utilized in the present screening procedure. A two stage protocol was adopted where cells were first grown in a chemically non-defined liquid medium (broth) after inoculation from a stock culture on solid medium. After harvesting the cells were resuspended in a small volume of the chemically defined transformation medium. The resuspended cells were then used as the inoculum for subsequent transformation study. This method prevents components of the broth from contaminating the

chemically defined transformation medium.

Transformations by fungi and bacteria have usually required the transformation substrate to be added after a long incubation period, up to 48 hours for *Cunninghamella* sp. (Gibson, 1984). These studies have also required an initial carbon source such as glucose to be depleted before substrate addition (Sewell, 1982). However, successful transformations of alkaloids by *Streptomyces* sp. have been obtained by substrate addition immediately after inoculation (Pan and Weisenborn, 1958) and this method was adopted for this study where the codeine substrate serves as the sole carbon source in the chemically defined medium. The chemically defined medium of Hug *et al.* (1974) was adapted for use in this study because of the fast doubling time reported for *Candida tropicalis* in this medium (Gmunder *et al* 1981).

Because of the very low yields of transformation products expected in screening studies, thin layer chromatography (TLC) and gas liquid chromatography (GLC) were used as the analytical techniques. TLC, although suffering from low resolution sensitivity, is a simple and quick method for identifying transformation products and was therefore initially used. GLC has been widely used for studying microbial transformation products (Yoshida and Aoyama, 1984). The GLC technique is more sensitive than high performance liquid chromatography (HPLC) and is flexible enough to detect various possible transformation products at very low concentrations. GLC was therefore used in all the culture studies in this section where

Derivatisation of the possible transformation products norcodeine and morphine was found to be unnecessary using an OV 17 column. The aim of the studies in this section was to identify strains of *Candida tropicalis* which are capable of N-demethylating and/or O-demethylating codeine.

2.2 Materials

2.2.1 Microorganisms

The following *Candida tropicalis* strains were investigated:

ATCC: 32113, 20221, 20336, 22577.

NCYC: 997.

Culture collections: ATCC: American Type Culture Collection, Maryland, USA.

NCYC: National Collection of Yeast Cultures.

2.2.2 Preparation and storage of stock cultures

The microorganisms were revived from their original freeze dried state in accordance with the instructions from the relevant cul;ture collection and stored under liquid nitrogen. The microorganisms were also transferred to Tryptone Soy Agar plates and incubated for 2 days at 37°C. The stock plates were maintained at 4°C and subcultured at intervals not exceeding 3 weeks.

2.2.3 Growth Media

Two types of media were used; a complex medium and a chemically defined medium.

i) Complex Media

a) Tryptone Soy Broth (TSB) (Oxoid)

This was prepared by adding 30 g of powdered medium to 1000 ml of glass distilled water. The mixture was heated to dissolve the powder and distributed into 150 ml glass bottles. The medium was sterilized by autoclaving at 121°C for 15 minutes.

b) Tryptone Soy Agar (TSA) (Oxoid)

32 g of powdered medium was added to 1000 ml of glass distilled water and thoroughly mixed. The solution was sterilized by autoclaving at 121°C for 15 minutes. Volumes (20 ml) were distributed into sterile plastic petri dishes and allowed to set. Set plates were stored for up to 14 days at 4°C. For viable counting purposes the plates were overdried for 20 minutes at 37°C.

ii) Chemically Defined Medium

The medium was adapted from that of Hug *et al*, 1974. The medium consisted of salts, trace elements, a yeast autolysate, vitamins and codeine as carbon source. All ingredients were of either Analar grade (BDH Ltd, Poole) or SLR grade (Fisons Scientific Apparatus,

Loughborough). The composition of the medium in g/l was as follows:

$(NH_4)_2SO_4$ (g/l) (10.0), $(NH_4)_2HPO_4$ (3.2),KCl (1.4), $MgSO_4.7H_2O$ (0.7) $CaCl_2.3H_2O$ (0.64), $FeCl_3.6H_2O$ (0.022), $MnSO_4.2H_2O$ (0.016), $ZnSO_4.7H_2O$ (0.014), $CuSO_4.3H_2O$ (0.0016), m-Inositol (0.093), Ca pantothenate (0.06), Thiamine HCl (0.009), Pyridoxine HCl (0.002), Biotin (0.0005) Yeast autolysate (3.0).

All ingredients were dissolved in glass distilled water in the order listed above and made up to 1000ml. Yeast autolysate was dissolved by gentle heating. The medium was then adjusted to pH 5-5.5 using dilute NaOH and 500ml volumes distributed into glass bottles. Sterilization was by autoclaving at 121°C for 15 minutes.

2.2.4 Test Substrates

Codeine phosphate (Macfarlane Smith Ltd, Edinburgh).
Norcodeine base and Norcodeine hydrochloride (Synthesized by M. Gibson, University of Bath).
Morphine sulphate (Macfarlane Smith Ltd, Edinburgh).

2.2.5 Chemicals and reagents

Silica gel F 254 TLC plates; Prespread 20 cm x 20 cm, 0.25 mm thickness (Merck) BDH Ltd, Poole.

Silica gel 600 TLC plates; 30 g silica gel 600 (Merck), BDH Ltd, was homogenised with 60 ml distilled water. The slurry was spread

over six 20 x 20 cm glass plates at a thickness of 0.25 mm. Plates were dried and stored in a dessicator at 20°C.

2.2.6 Solvents

a) For extraction: 2-methyl propanol and 1,2-dichloroethane (HPLC grade). (Aldrich), and chloroform, laboratory reagent grade, (Fisons).

b) For reconstitution of chromatographic samples: methanol and tetrahydrofuran (THF), HPLC grade, (Fisons Scientific Apparatus). Because THF was unstabilized, it was filtered before use through Alumina Woelm BAKT 1 (Woelm Pharma GmbH, Eschwerge.) to remove peroxide formed during storage.

2.2.7 Equipment and Instrumentation

Analytical balance; Oertling series 040 5 figure balance.
Autoclave bags; DRG Ltd, Bristol.
Centrifuge; MSE High Speed 18, MSE Scientific Instruments, Crawley.
Centrifuge bottles; 250 ml polypropylene, MSE Scientific Instruments, Crawley.
Chart recorder; Servogor 120, BBC Gorez Metrawatt, Austria.
Culture flasks; Glass Erlenmeyer flasks, 250 ml, metal capped, Pyrex.
Gas Liquid Chromatograph; Perkin Elmer FII, Series 2, fitted with flame ionisation detector, Perkin Elmer, Beaconsfield.
GLC column; 2m glass, 4mm i.d., packed under nitrogen with 3% OV17 on Chromosorb WHP 80/100 mesh (5058P), Phase Separation Ltd,

Queensferry, Clwyd.

GLC syringes; 5 µl and 10 µl volumes,calibrated in 0.1 µl divisions, fieeted with 70 mm dome tipped needles, Scientific Glass Engineering U.K. Ltd., London.

Gilson pipettes; variable range (Pipetman 0.2 to 1.0 ml and 1 to 5 ml), Gilson Medical Electronics, France.

Membrane filter units; Swinnex units, 25 mm and 47 mm, Millipore Corporation, Massachussetts, USA.

Petri dishes; Sterilin single vent 90 mm, Sterilin.

Pipette tips; Plastic PVC supplied by Fisons Scientific Apparatus.

pH meter; PW 9418 model, Pye Unicam Ltd, standardised with buffer solutions of pH 4.0 and 9.0.

Rotary evaporator; Buchi, Fisons Scientific Apparatus.

Rotary Incubator; New Brunswick G-25R cooled, gyratory with 1" stroke. New Brunswick Scientific Co. Inc., New Jersey, USA.

Spectrophotometer; SP600 model, Pye Unicam Ltd, with 1 cm matched glass cuvettes.

All glassware was washed and brushed clean in Link Det (Link Chemicals Ltd., London) and soaked in glass distilled water for 12 hours. Glassware was then rinsed throughly and sterilised by dry heat at 180°C for 60 minutes. Reusable plasticware eg. pipette tips were washed and brushed clean in Link Det and thoroughly rinsed with tap water and then with distilled water. After soaking in distilled water for 12 hours the plasticware was dried at 45°C. Plasticware was subsequently sterilised by autoclaving at 121°C for 15 minutes.

2.3 Methods

2.3.1. Growth Conditions and Incubation of Cultures

The test organisms were grown in liquid media using a 2 stage protocol. A volume (100 ml) of Tryptone Soy Broth was placed in a 250ml metal capped Erlenmeyer flask and inoculated with a single colony of appropriate organism from a stock culture on Tryptone Soy Agar using a wire loop. This primary culture was incubated for 18 hours at 30°C in a shaking incubator (150 rpm). The primary culture was then centrifuged for 5 min at 3500 rpm. The cells were resuspended into chemically defined growth medium (100 ml). This suspension was used to inoculate (10 ml) a second flask containing chemically defined growth medium (prewarmed) containing codeine (1.0 mM) as codeine phosphate. The secondary culture was then incubated at 30°C with shaking (150 rpm) for 12 hours. In transformation experiments, codeine was the sole carbon source. The codeine phosphate was added as a filter sterilized solution in distilled water to give the required concentration.

2.3.2. Viable Count Determination

It was necessary to establish a method for assessing growth of the test organisms during codeine transformation studies. A viable counting technique to assess growth was thought suitable because the test organisms grew as discrete colonies on the surface of Tryptone Soy Agar plates. Microscopic examination of diluted cultures showed

the presence of individual yeast cells and it was assumed that a colony on solid medium would have arisen from a single viable cell.

A serial dilution and surface spread technique was used to determine the number of viable organisms in a culture. A 0.1 ml sample of the culture was diluted in 9.9 ml of sterile glass distilled water and thoroughly mixed using a Whirlimixer. Further 1 in 10 dilutions were then performed with thorough mixing to give a final dilution containing 250-750 viable organisms per ml. A 0.2ml sample of the final dilution was placed on the surface of each of five overdried TSA plates and spread evenly using a sterile glass spreader. The suspension was allowed to soak into the solid medium and the plates were then inverted and incubated for 48 hours at 30°C. The colonies on each plate were counted after incubation and the number of viable organisms in the original suspension was calculated from the mean count of five replicate plates.

It was necessary to establish that the surface spread technique was capable of detecting, within limits of normal sampling error, comparable numbers of viable organisms per unit volume in a number of samples taken from a single yeast culture. Therefore a 100ml secondary stage culture of *Candida tropicalis* ATCC 32113 in chemically defined medium was set up as in Section 2.3.1. The culture was incubated at 30°C for 24 hours. Five 0.5ml samples were then taken and each was serially diluted through five 1 in 10 dilutions in sterile glass distilled water. Volumes (0.2ml) of each of the final dilutions were then pipetted and spread onto five TSA plates. The

plates were then incubated for 48 hours at 30°C and colonies counted. The results and statistical analysis of data are shown in Table 2.1. The analysis shows that the variation between the samples is not significantly greater than the variation within the samples. Furthermore, since the overall coefficient of variation is less than 5%, it was concluded that the viable count technique was reproducible and suitable for use in subsequent experiments.

2.3.3 Optical density and viable count correlation

Although the surface spread technique was an accurate method of determining the viability of *Candida tropicalis* suspensions,there was a 48 hour delay before the results were known. The optical density of a suspension of unicellular organisms is a function of the total number of organisms in the suspension. Under defined conditions, the optical density can also give a rapid estimate of the viable count of the suspension. Therefore correlations of viable counts of suspensions with optical density at 550 nm were carried out. It was found that at 550 nm the components of the chemically defined growth medium showed least absorbance. The *Candida tropicalis* suspensions, however, showed significant absorbance at 550 nm.

A 12 hour secondary stage culture (100 ml) of *Candida tropicalis* ATCC 32113 was prepared as in Section 2.3.1. Volumes (10 ml) of the growth culture were diluted with sterile glass distilled water to give an optical density of 0.6 at 550 nm. This culture was then further diluted with sterile glass distilled water to give a range of dilutions each with a different optical density in the range 0.1-0.6.

Sample	Dilution factor	Colony counts					Mean count	Mean viable count/ml
1	1×10^5	57	55	61	56	56	56·2	$2{\cdot}81 \times 10^7$
2		54	50	57	55	56		
3		58	57	56	57	51		
4		54	59	58	61	59		
5		53	53	60	55	58		

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F
Between samples	4	38·64	9·66	1·45
Within samples	4	43·28	10·82	1·62
Residual	16	106·64	6·665	
Total	24	188·56		

Overall coefficient of variation (C.V.) = 4·6%

$F(4,16)\ P_{0{\cdot}05} = 3{\cdot}01$

Table 2·1 Reproducibility of viable counts for Candida tropicalis ATCC 32113 with statistical analysis of data

The optical density of each dilution was read at 550 nm against the appropriate dilution of the growth media (sterile) as the blank. Each dilution was then further serially diluted in glass distilled water to obtain the viable count. 0.2 ml volumes of the appropriate dilutions were plated onto overdried TSA plates and incubated for 48 hours at 30°C.

The viable counts and statistical analysis of the data are shown in Table 2.2. Viable counts are plotted as a function of the corresponding optical density at 550nm in Figure 2.1. The statistical analysis showed there was a significant linear correlation between optical density at 550nm and viable count per ml over the range 0.1 to 0.6. Therefore optical density measurements at 550nm could be used as a rapid estimate of the viability of *Candida tropicalis* suspensions in subsequent experiments.

2.3.4. Growth Curves

2.3.4.1. Construction of Growth Curves

Experiments were conducted to determine whether optical density measurements could be used to speed up the process of consturcting growth curves. A primary culture (100 ml) of *Candida tropicalis* ATCC 32113 was set up as in Section 2.3.1. The primary TSB culture, after 18 hour incubation, was diluted to give an optical density of 0.6 at 550 nm. A volume (10 ml) of the diluted primary culture was then used to inoculate 90 ml of secondary culture containing chemically

Sample No.	Optical Density 550nm.	Mean Colony Count	Dilution Factor	Viable Count per ml.
1	0·6	92	10^{-4}	$4{\cdot}6 \times 10^{6}$
2	0·41	66	10^{-4}	$3{\cdot}2 \times 10^{6}$
3	0·33	47	10^{-4}	$2{\cdot}35 \times 10^{6}$
4	0·22	160	5×10^{-4}	$1{\cdot}6 \times 10^{6}$
5	0·19	24	10^{-4}	$1{\cdot}2 \times 10^{6}$
6	0·09	92	5×10^{-3}	$9{\cdot}2 \times 10^{5}$

Correlation coefficient	0·984
Gradient	1·152
Intercept	0·038
Standard error of gradient	0·955

Table 2·2 Data and statistical analysis for optical density at 550nm against viable count ml^{-1} for suspensions of Candida tropicalis ATCC 32113

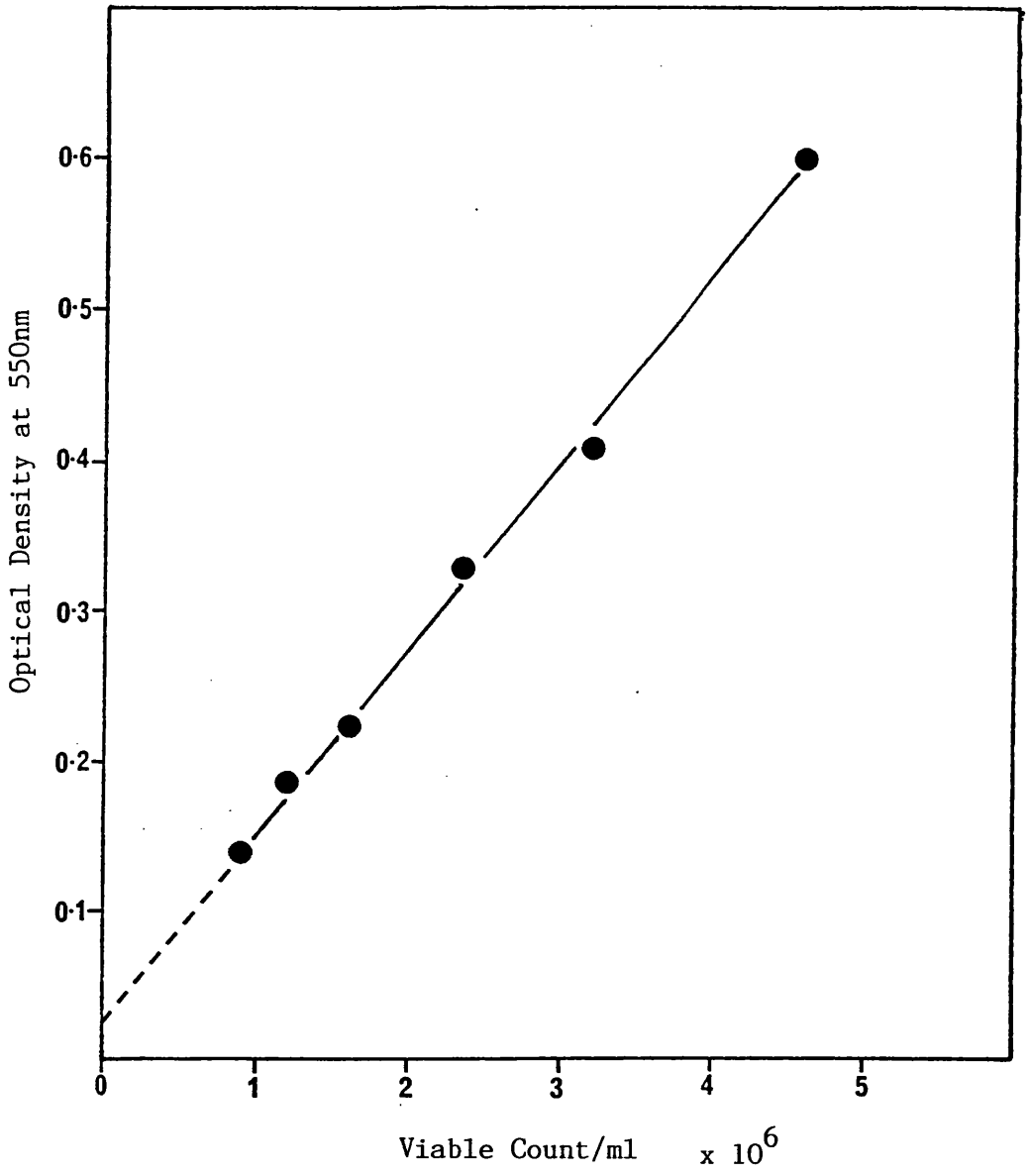


Figure 2·1 Plot of optical density at 550nm against viable count/ml for samples of a 12 hour secondary culture of Candida tropicalis ATCC 32113 grown on glucose(1%) in chemically defined growth medium.

defined growth medium with 1% glucose and incubated with shaking at 30°C. After inoculation and mixing a sample of the secondary culture was taken at time 0 and the optical density measured at 550 nm, using sterile pre-warmed chemically defined growth medium as blank. Further samples were taken at 1 hour intervals during incubation and the optical density at 550 nm and the viable count of each sample was determined. Samples with optical density greater than 0.8 were diluted with chemically defined growth medium to obtain values in the range 0.1-0.6. The results are given in Table 2.3 and illustrated in Figure 2.2.

The growth curves of *Candida tropicalis* ATCC 32113 over the 12 hour incubation period when constructed using optical density measurements and viable counts show very different characteristics; the initial lag phase was more pronounced using optical density measurements and the beginning of the stationary phase apparent with viable counts was not seen using optical density measurements. It was therefore concluded that optical density measurements were not suitable for constructing growth curves and therefore viable count determinations were used in all subsequent experiments.

2.3.4.2. Reproduceability of Growth Curves

Because growth characteristics and parameters were to be used in subsequent experiments, it was necessary to show that the growth of the organisms in replicate secondary cultures was reproducible within limits of normal error.

Sample No.	Incubation Time (hr)	Optical Density 550nm	Viable Count per ml.
1	0	0·121	$1·36 \times 10^5$
2	1	0·127	$1·54 \times 10^5$
3	2	0·131	$1·89 \times 10^5$
4	3	0·135	$2·32 \times 10^5$
5	4	0·151	3.1×10^5
6	5	0·185	$4·61 \times 10^5$
7	6	0·226	$7·63 \times 10^5$
8	7	0·310	$1·65 \times 10^6$
9	8	0·50	$3·42 \times 10^6$
10	9	0·762	-
11	10	0·121	$6·41 \times 10^6$
12	11	-	$7·4 \times 10^6$
13	12	0·27	$8·1 \times 10^6$

Table 2·3 Optical density and viable count data for samples taken at different times during incubation of a suspension of *Candida tropicalis* ATCC 32113 at 30°C

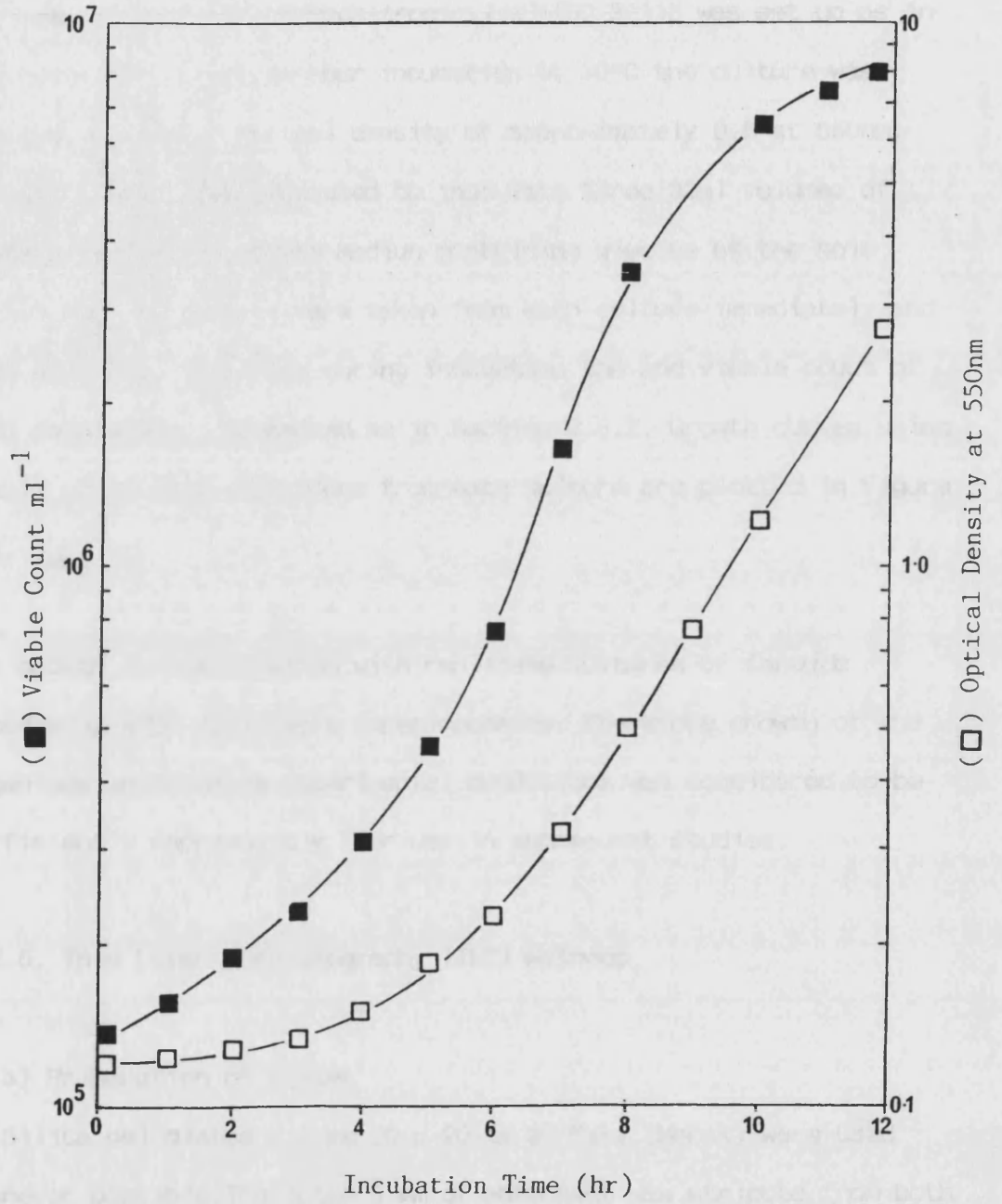


Figure 2·2 Growth curves for Candida tropicalis ATCC 32113 plotted using optical density measurements at 550nm (□) and viable count ml^{-1} (■). Incubation temperature 30°C.

A primary culture of *Candida tropicalis* ATCC 32113 was set up as in Section 2.3.2. After 18 hour incubation at 30°C the culture was diluted to give an optical density of approximately 0.6 at 550nm. Volumes (10ml) were then used to inoculate three 90ml volumes of chemically defined growth mediun containing glucose as the sole carbon source. Samples were taken from each culture immediately and then at 1 hour intervals during incubation the and viable count of each sample was determined as in Section 2.3.2. Growth curves using viable count data determined from each culture are plotted in Figure 2.3.

The growth curves obtained with replicate cultures of *Candida tropicalis* ATCC 32113 were superimposable. Therefore growth of the organisms under these experimental conditions was considered to be sufficiently reproducible for use in subsequent studies.

2.3.5. Thin Layer Chromatography (TLC) Methods

a) Preparation of plates

Silica gel plates 0.2 mm 20 x 20 cm 60 F_{254} (Merck) were used whenever possible.The outer 5 mm of absorbant was stripped from both sides of each plate to ensure an even solvent front. The plates were then stored at 120°C for 30 minutes in an oven prior to use.

b) Sample application

Reconstituted extract samples and reference compounds were applied

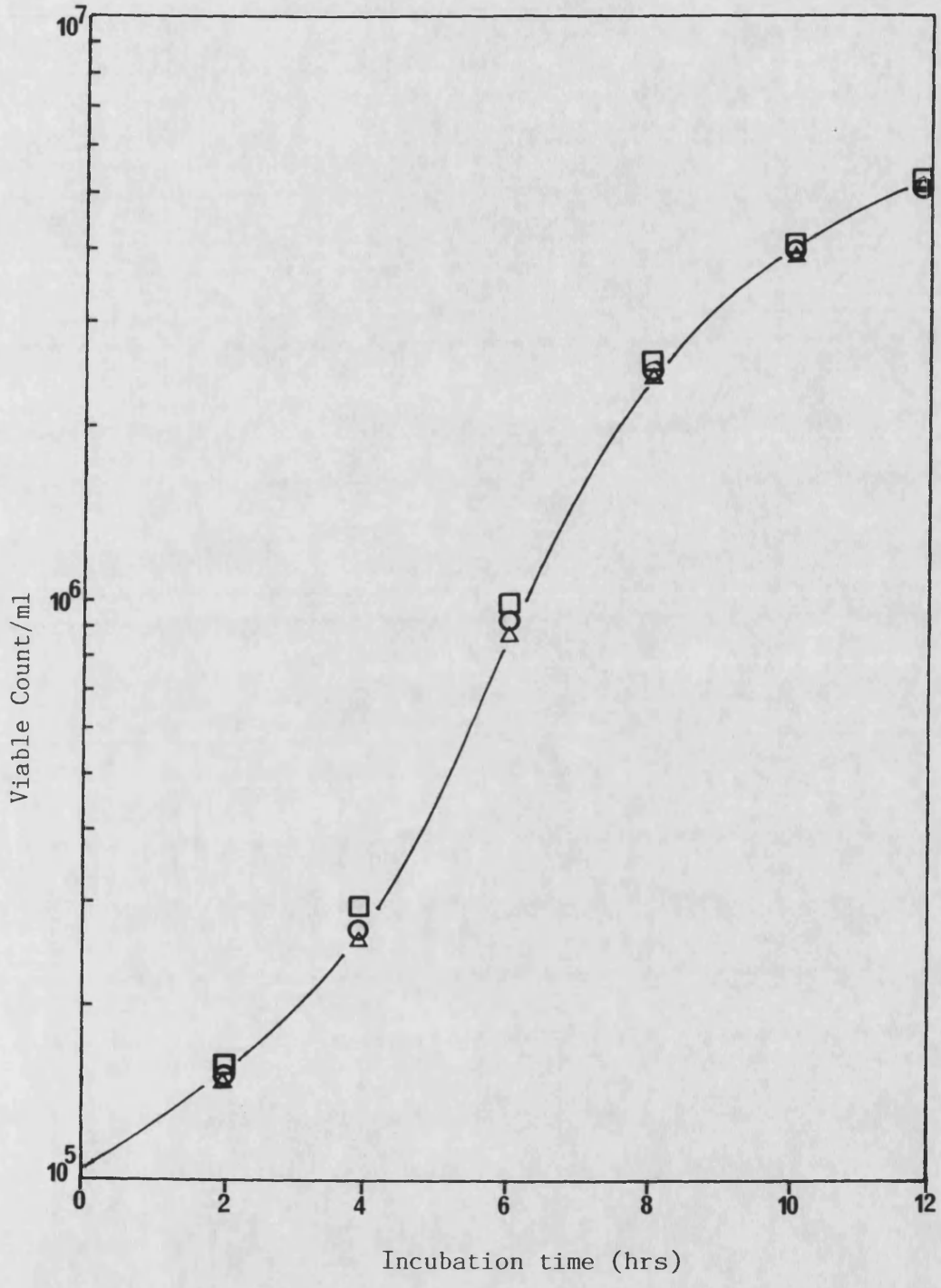


Figure 2·3 Reproducibility of growth curves for Candida tropicalis ATCC 32113 using viable count determination.
Incubation temperature 30°C.

to the plates using the appropriate reconstitution solvent. 5 µl volumes of each sample were applied using a glass capillary tube. Samples were applied 20 mm from the bottom of the plate. To prevent band broadening the solvent was evaporated from the plate between each application. Each band consisted of several layers applied sequentially.

c) Development of plates

200 ml of mobile phase solvent was added to a chromatography tank which was sealed and allowed to equilibrate for at least 4 hours. TLC plates, after sample application and evaporation of the solvent, were then lowered into the tank which was resealed. The solvent front was then developed for 10-15 cm at room temperature.

d) Mobile phase

The mobile phase used consisted of ;

Methanol	130 ml
Toluene	17 ml
n-butanol	26 ml
Triethylamine	1 ml
Distilled water	26 ml

e) Visualisation

The plates were first examined under 254 nm UV light where the eluted components showed up as fluorescent spots. The plates were then sprayed with a 10% sulphuric acid solution and placed in an oven at 120°C until visualised.

f) Treatment of TLC results

The migration of sample components is expressed as R_f values;

$$R_f = \frac{\text{Distance travelled by component (cm)} \times 100}{\text{Distance travelled by solvent front (cm)}}$$

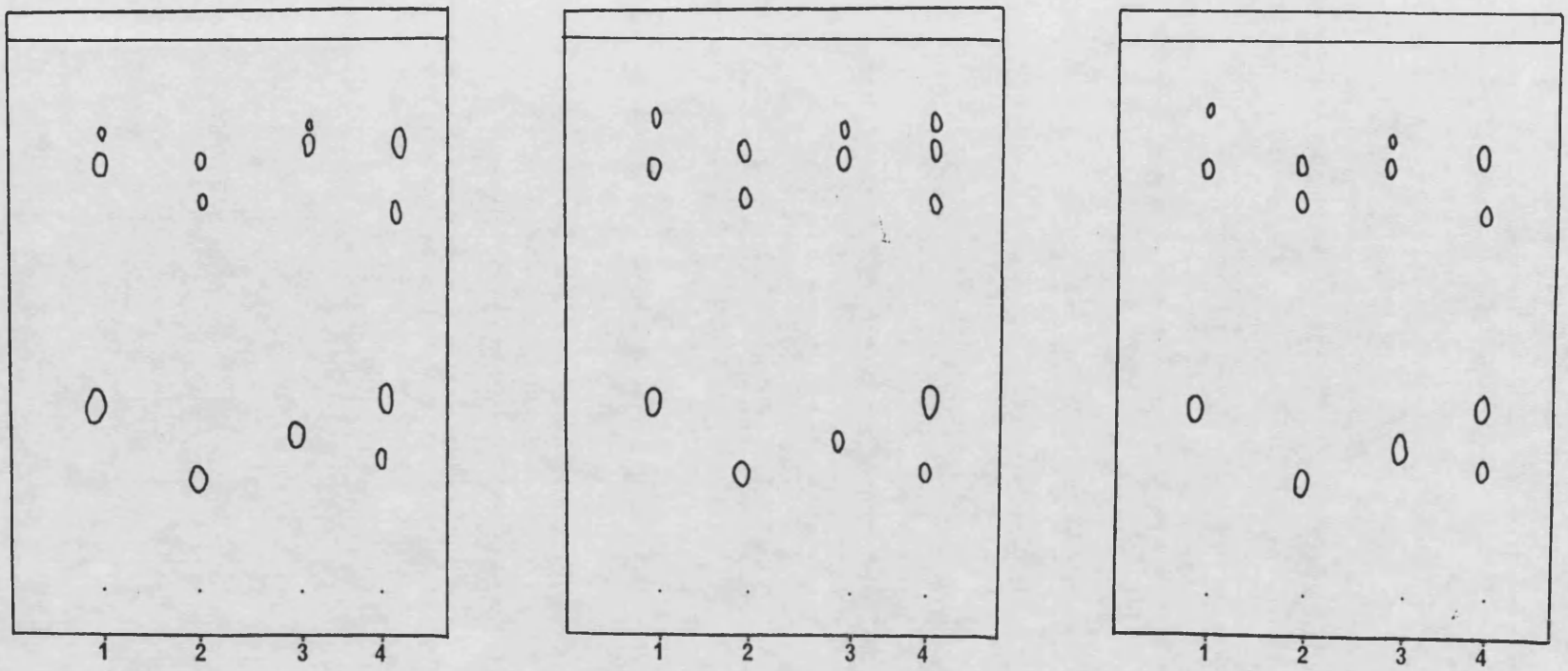
Two values for R_f were recorded for each component, one for the upper limit and one for the lower limit of the spot.

g) Reproducibility of TLC results

To account for any variation in the R_f values of the same samples tested on different TLC plates, it was necessary to determine the reproducibility of the TLC method. Three plates of the same dimensions were prepared and the samples listed in Table 2.4 were applied to each plate. The plates were then developed and visualised as described above. The data obtained are shown in Table 2.4. The standard solutions of codeine, norcodeine and morphine all produced one major spot towards the bottom of the plate and two minor spots nearer the solvent front (Figure 2.4). The R_f values of the minor spots were inconsistent from chromatogram to chromatogram and were assumed to be impurities in the samples. Therefore these minor spots were ignored when calculating R_f values. The R_f values quoted in Table 2.4 were calculated for the major spots only and the values for each sample were very similar. It was therefore concluded that the TLC method was sufficiently reproducible for rapid analysis of transformation mixtures.

	R_f Values		
	Plate 1	Plate 2	Plate 3
Codeine standard	35·7–40·5	36·5–41·2	35·7–41·7
Norcodeine standard	23·8–27·9	24·8–30·1	23·5–27·6
Morphine standard	31·5–35·7	30·8–34·3	29·4–34·1
Transformation mixture	37·5–42·3	36·7–42·0	36·5–40·6
	27·9–30·9	25·4–28·4	25·9–29·4

Table 2·4 Ranges of R_f values from replicate TLC chromatograms using standard solutions of codeine, norcodeine, morphine and transformation mixture of Candida tropicalis ATCC 32113



1. Codeine standard
2. Norcodeine standard
3. Morphine standard
4. Transformation mixture

Figure 2.4 Reproducibility of TLC chromatograms using standard solutions of codeine, norcodeine and morphine and transformation mixture of *Candida tropicalis* ATCC 32113

2.3.6. Gas-Liquid Chromatography (GLC) Methods

a) Glass column preparation

A freshly packed column was first conditioned to remove impurities and settle the packing material. Columns were packed with a 3% OV-17 stationary phase on Chromosorb WHP 80/100 mesh and conditioned in the GLC oven. The column was disconnected from the detector and conditioned at 275°C with passage of nitrogen gas at 10 ml min^{-1} for 12 hours.

b) Supply of gases to chromatograph

The gases were supplied through PTFE tubing (0.25" diameter) to a pressure regulator at 55 psi. Prior to delivery to the chromatograph, the pressure was reduced using the regulator to obtain the required flow rate.

Nitrogen gas (carrier) : flow rate of 120 ml min^{-1}

Hydrogen : 20 psi Air : 25 psi

c) Injection of samples

Extracted products and reference standards were dissolved in the methanol solvent. 2 µl of the sample solution was drawn into a 10µl syringe. The rubber septum at the column head was pierced by the syringe needle which was inserted into the top 1 cm of the column packing for injection. Between sample injections the syringe was thoroughly rinsed with methanol. To account for artefacts arising due to sample contamination, control extracts were analysed, followed by transformation mixture extracts and finally the reference

samples.

d) Chromatograph operation

i) Detector sensitivity; the ionisation amplifier determined the sensitivity which was variable between 2 x 10^5 and 1. The optimum setting was found to be 1 x 10^3.

ii) Oven temperature; the optimum oven temperature was one that produced base line separation of all sample components in the shortest analysis time. This was determined for the test substrate,codeine, the major transformation product, norcodeine, and another possible product morphine by obtaining chromatograms at oven temperatures of 210, 230, 250, and 270°C (Figure 2.5) The gas flow also needed variation for optimal separation, and a flow of 120 ml min^{-1} was found to be suitable for all conditions. From the chromatograms obtained (Figure 2.5) an oven temperature of 250°C was selected for all subsequent GLC analyses.

iii) Recorder chart speed; this was set at 0.5mm/min to optimise definition of peaks from the chromatogram.

The final chromatogram conditions used for analysis are listed in in the table below:

Test substrate/transformation product : codeine/norcodeine, morphine/normorphine

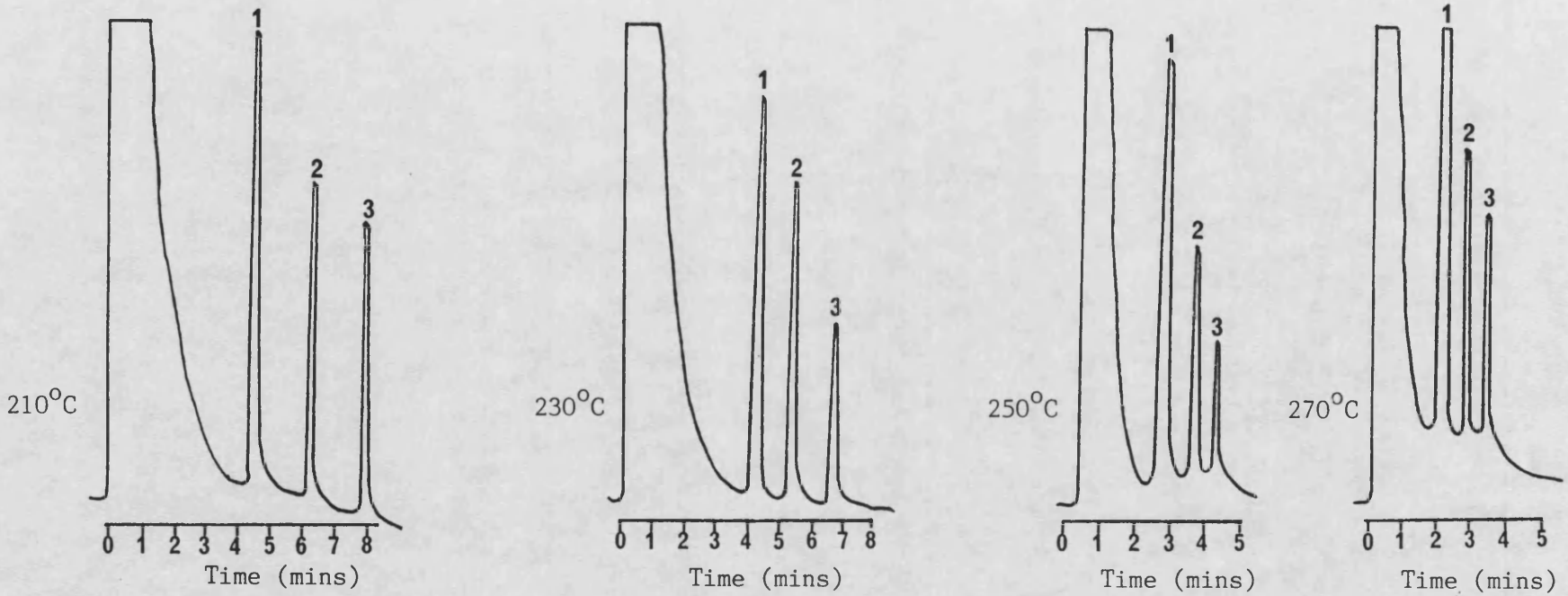
Oven temperature : 250°C

Detector temperature : 300°C

Carrier gas flow : 120 ml/min

Solvent : THF

Column : OV-17 2 metre on Chromosorb WHP 80/100 mesh (5058P)



1. Codeine standard
2. Morphine standard
3. Norcodeine standard

Figure 2·5 Typical GLC chromatograms for standard solutions of codeine, norcodeine and morphine at various oven temperatures

e) Treatment of results

The performance of the GLC column will vary from day to day and this will cause variation in the retention time of the sample. However, changes in column performance will affect all samples equally. Therefore there will be no change in the relative retention times of an injected multi-component sample. In this study all retention data are reported as the Relative Retention Time (RRT) which was calculated relative to the retention time of codeine.

$$RRT = \frac{\text{Retention time of component (c)}}{\text{Retention time of codeine}}$$

where component c may be the reference compound, test substrate or transformation product.

Components which eluted rapidly and formed peaks with the solvent front peak were ignored. Peaks of less than 10 mm were considered too small for analysis and not plotted.

2.3.7. Suitability of Extraction Procedure

Before conducting the qualitative screening experiments, it was necessary to establish that the extraction procedure was capable of extracting all transformation products. The following procedure was used for extracting transformation mixtures. The mixture was vigorously stirred with 100ml of extraction solvent consisting of 10% 2-methylpropanol in 1,2-dichloroethane. The aqueous layer was adjusted to pH 11-11.5 with dropwise addition of NaOH solution. After gentle mixing in a separating funnel the organic phase was drawn off.

The aqueous phase was twice extracted with 50ml portions of extraction solvent. The combined extracts were dried using anhydrous $MgSO_4$ and filtered under vacuum. The solvent was evaporated in a rotary evaporator under reduced pressure over a water bath at 55°C. The residue was cooled and dissolved in 2.0ml of reconstitution solvent consisting of THF and methanol (1:1). The following procedure was used to assess the suitability of the extraction method; to a flask containing 100ml of chemically defined growth medium was added codeine,norcodeine and morphine and codeine N-oxide to final concentrations of 1mM. After extraction as described the residues were analysed by GLC as in Section 2.3.7. The production of peaks with a height of 10mm or greater corresponding to a transformation product showed that the extraction procedure and GLC sensitivity were sufficient to detect transformation. The extraction procedure produced peaks of 20-30mm with detector sensitivity at 1×10^3 (Figure 2.5). Therefore the extraction method was considered to be suitable for qualitative analysis in subsequent experiments.

2.4. Experimental

2.4.1 Effect of Presence of Codeine on Growth Characteristics

Prior to any transformation experiments, it was necessary to establish the effect of the presence of codeine as the sole carbon source on the growth characteristics of the *Candida tropicalis* strains. The

Candida tropicalis strains used for testing were NCYC 997,ATCC 20221, 20336, 22577 and 32113. A primary culture of each of these was set up as in Section 2.3.1. After 18 hours incubation the primary cultures were each centrifuged (3500 rpm, 5 min), resuspended in chemically defined growth medium and diluted to an OD_{550} of approx. 0.6. The diluted culture was then used to inoculate secondary cultures in chemically defined growth medium containing 1% glucose as the sole carbon source. This was incubated with shaking at 30°C. Samples were taken immediately after inoculation and then at hourly intervals for viable count determination.

For each *Candida tropicalis* strain a duplicate secondary culture was set up as above, but in chemically defined growth medium containing 1.0 mM codeine. Control cultures were also set up with chemically defined growth medium containing no carbon source.

Growth curves were constructed for the *Candida tropicalis* strains from viable count determinations and these are plotted in Figures 2.6-2.8. Growth was observed with all strains of *Candida tropicalis* with glucose as the sole carbon source. However, growth was observed in *Candida tropicalis* strains NCYC 997, ATC 22577 and 32113 only with codeine as the sole carbon source.

The strains grown on codeine showed an increase in initial lag time and the number of viable cells at the end of the incubation period was lower than growth with glucose. However, it was concluded that the presence of 1mM codeine as sole carbon source produced satisfactory growth of *Candida tropicalis* NCYC 997, ATCC 22577 and 32113, and that these strains were suitable for further screening

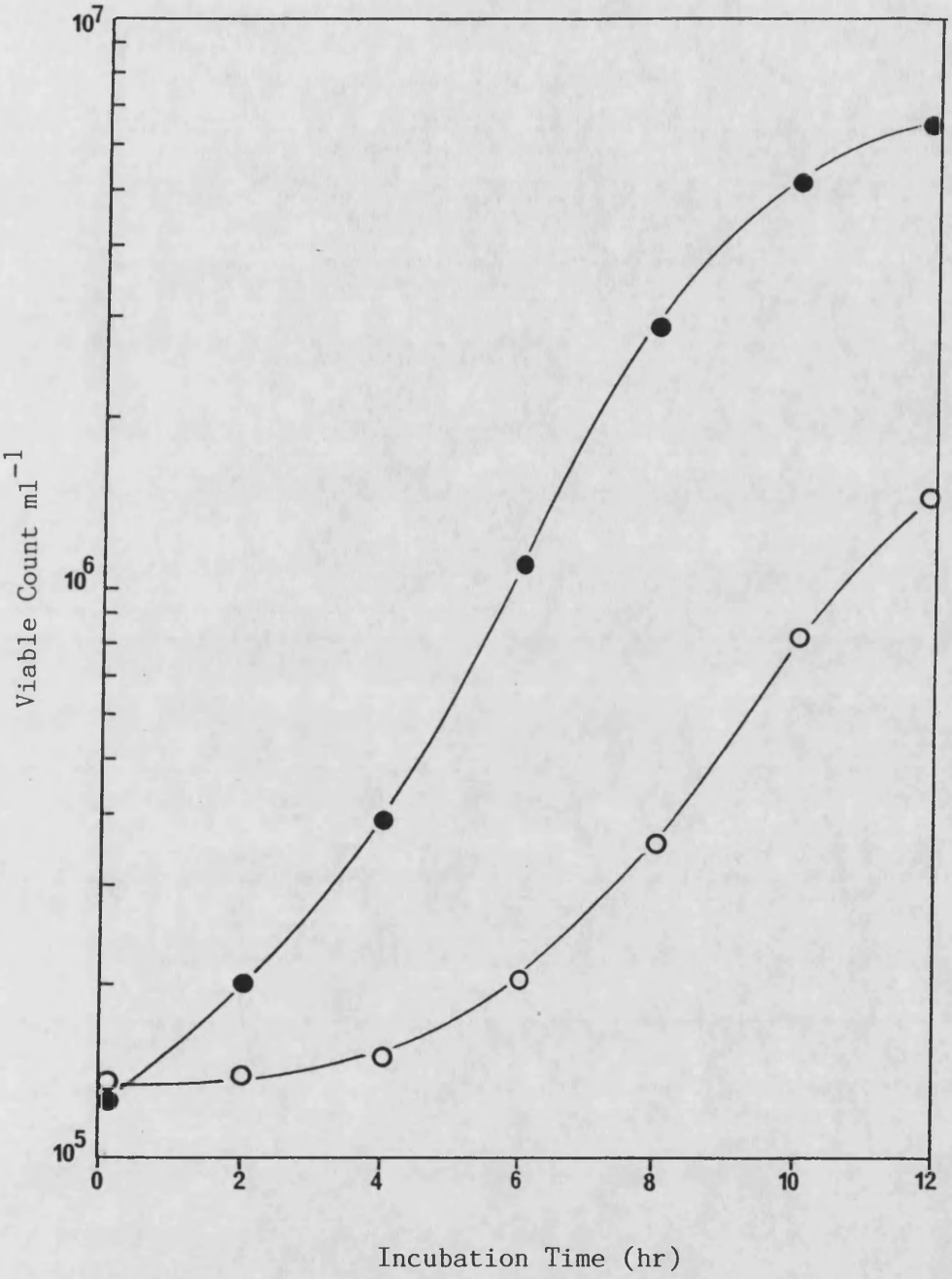


Figure 2·6 Viable count/ml plotted against incubation time for a secondary culture of Candida tropicalis NCYC 997 grown in presence of glucose(●) and presence of codeine(O) as sole carbon sources. at 30°C.

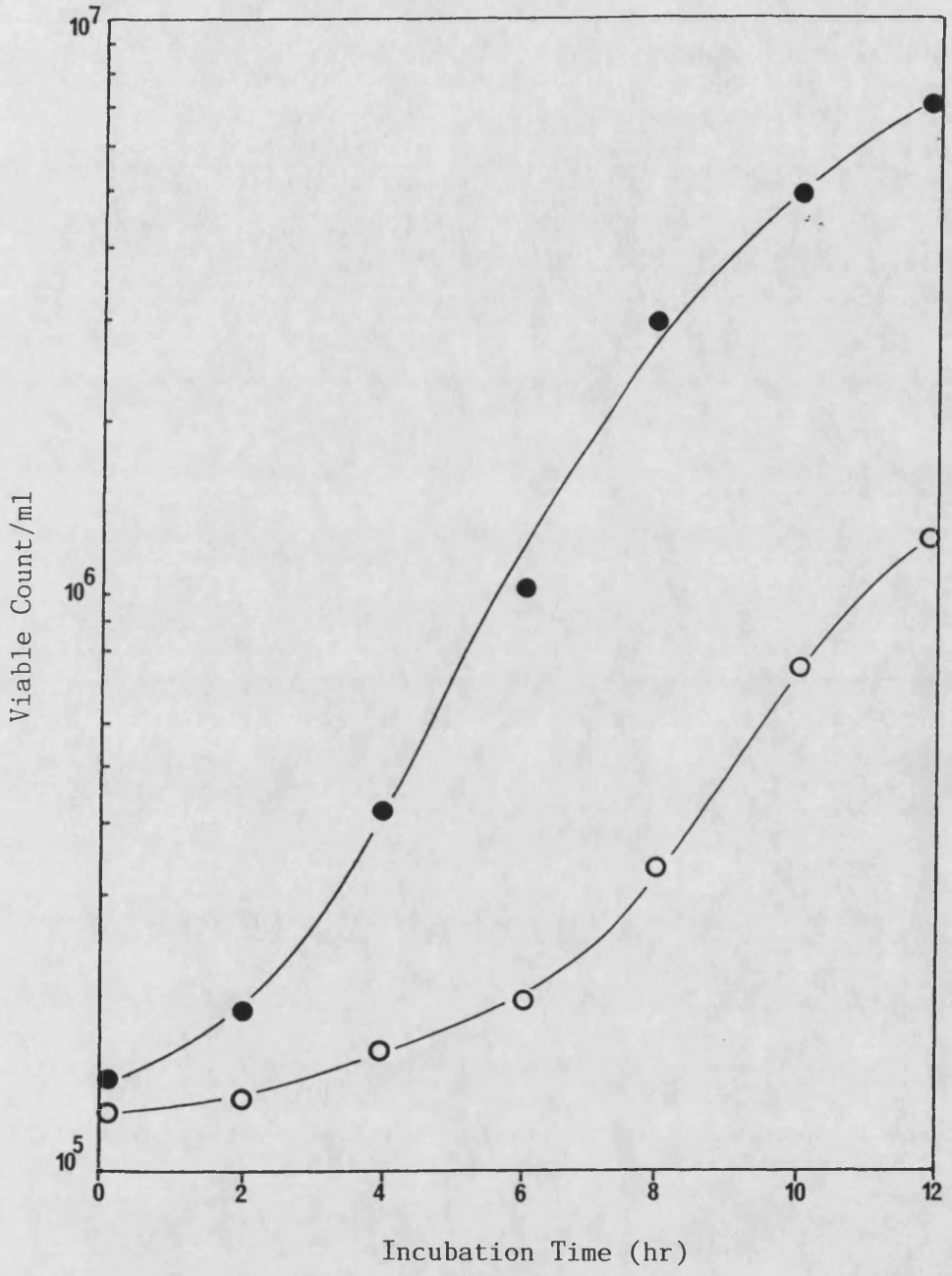


Figure 2·7 Viable count/ml plotted against incubation time for a secondary culture of Candida tropicalis ATCC 22577 grown in presence of glucose (●) and presence of codeine(○) as sole carbon source at 30°C.

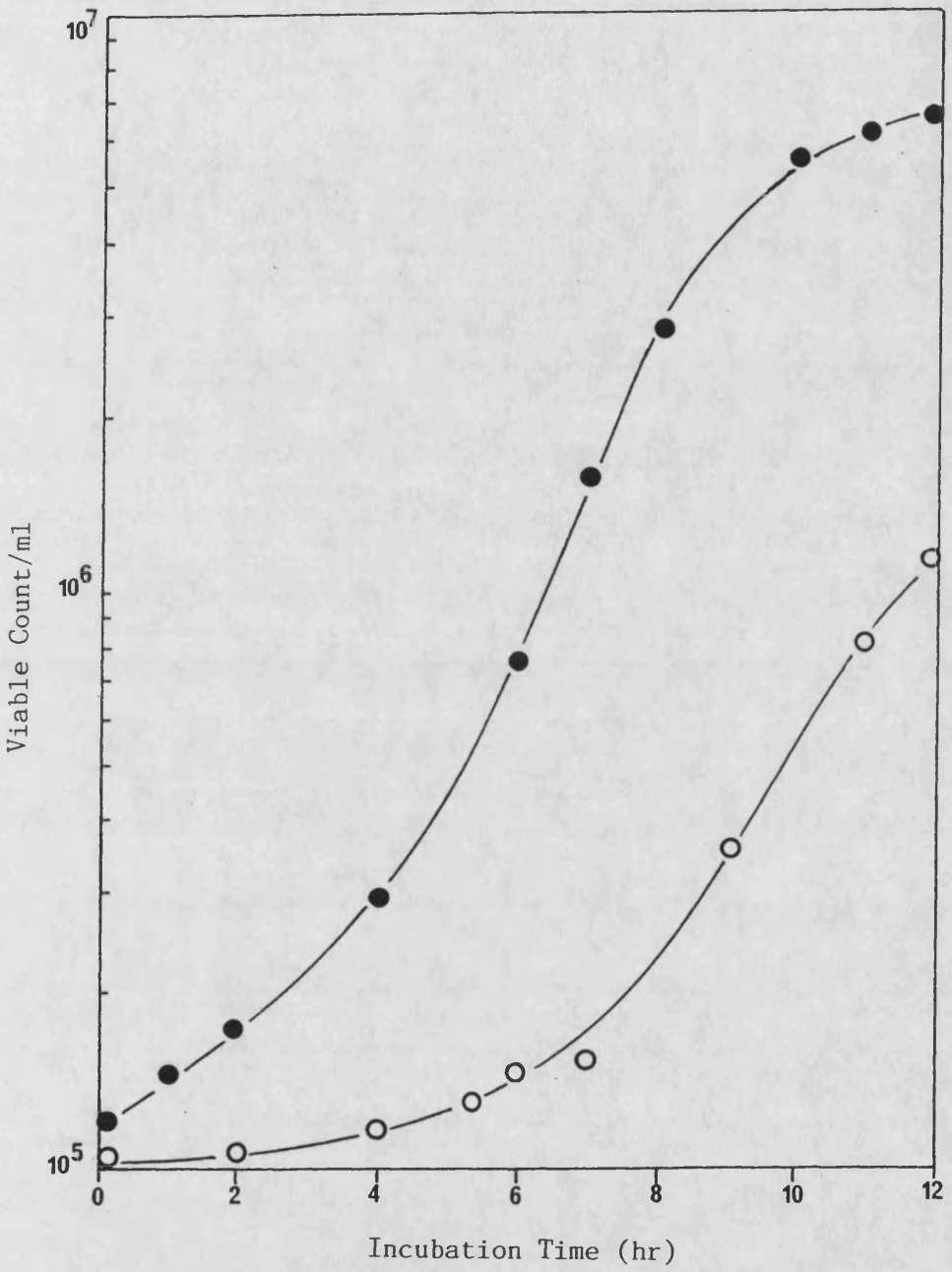


Figure 2·8 Viable count/ml plotted against incubation time for a secondary culture of Candida tropicalis ATCC 32113 grown in presence of glucose(●) and presence of codeine(○) as sole carbon sources. at 30°C.

studies.

2.4.2. Screening of *Candida tropicalis* strains for N-demethylation activity

For transformation studies the same two stage process as above was used. A primary culture was produced , and after centrifugation, resuspension and dilution, was used to inoculate a secondary culture of prewarmed chemically defined growth media containing the codeine as the sole carbon source. A similar procedure has been used previously (Rosazza and Smith, 1979; Sewell, 1984) for *Cunninghamella* sp. but the intermediate step of centrifugation and resuspension of microbial growth before inoculation was not carried out.

Flasks of 100ml of chemically defined growth medium containing 1mM codeine as codeine phosphate were inoculated with 10 ml of a diluted (OD_{550} 0.6) resuspended 18 hour primary culture of each strain of *Candida tropicalis*. Transformation cultures were then incubated at 30°C with rotary shaking (180 rpm) for 24 hours.

For each strain of *Candida tropicalis* tested, duplicate transformation mixtures were set up. The following control flasks were also prepared and incubated under the same conditions:

a) sterile chemically defined growth medium (100 ml) containing 1 mM codeine as codeine phosphate.

b) inoculated secondary culture in chemically defined growth medium containing no carbon source.

After incubation each flask was subjected to TLC analysis and extracted prior to GLC analysis as in Section 2.3.7.

The typical thin layer chromatograms obtained from analysis of

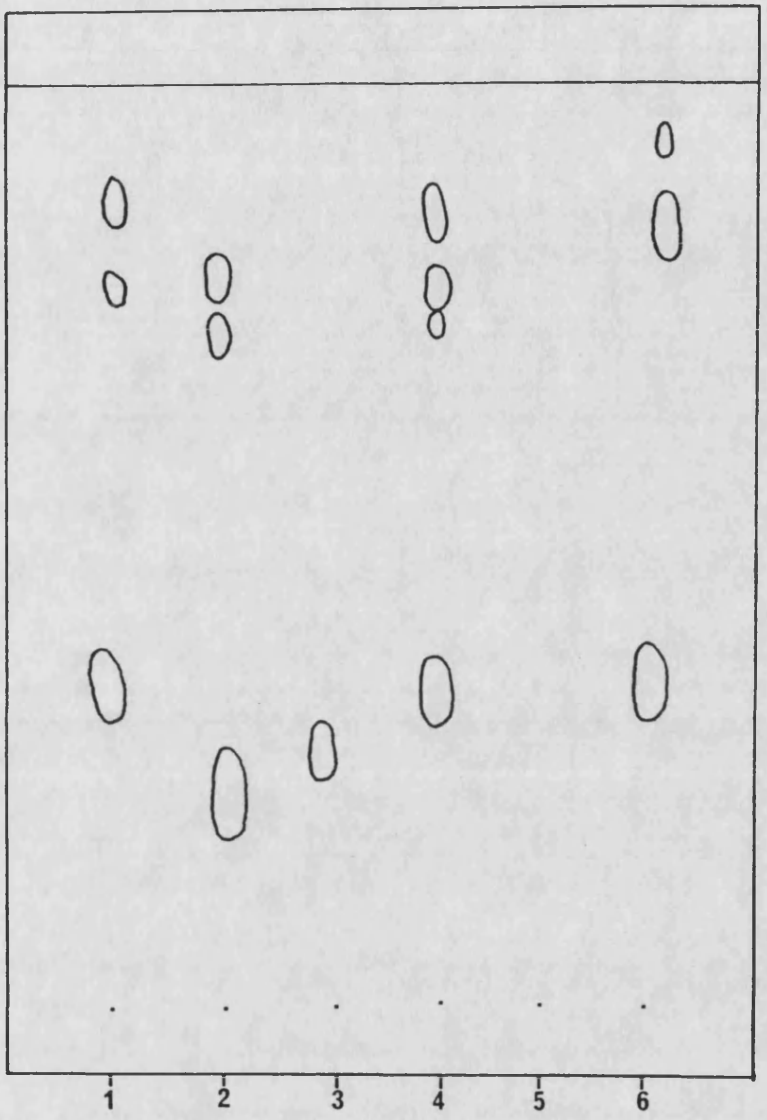
transformation mixtures for each of the five *Candida tropicalis* strain are shown in Figures 2.9-2.13.

The TLC migration data for each strain are shown in Table 2.5. The GLC chromatograms obtained for transformation extracts for each strain are shown in Figures 2.14-2.18 and the retention data summarised in Table 2.6.

Analysis of transformation extracts by GLC showed that *Candida tropicalis* strains NCYC 997 and ATCC 22577 produced both norcodeine and morphine as the transformation products. *Candida tropicalis* ATCC 32113 produced only norcodeine as a transformation product. It was therefore concluded from the screening studies that only *Candida tropicalis* ATCC 32113 was capable of exclusive N-demethylation of codeine. This strain was therefore used in the subsequent experiments.

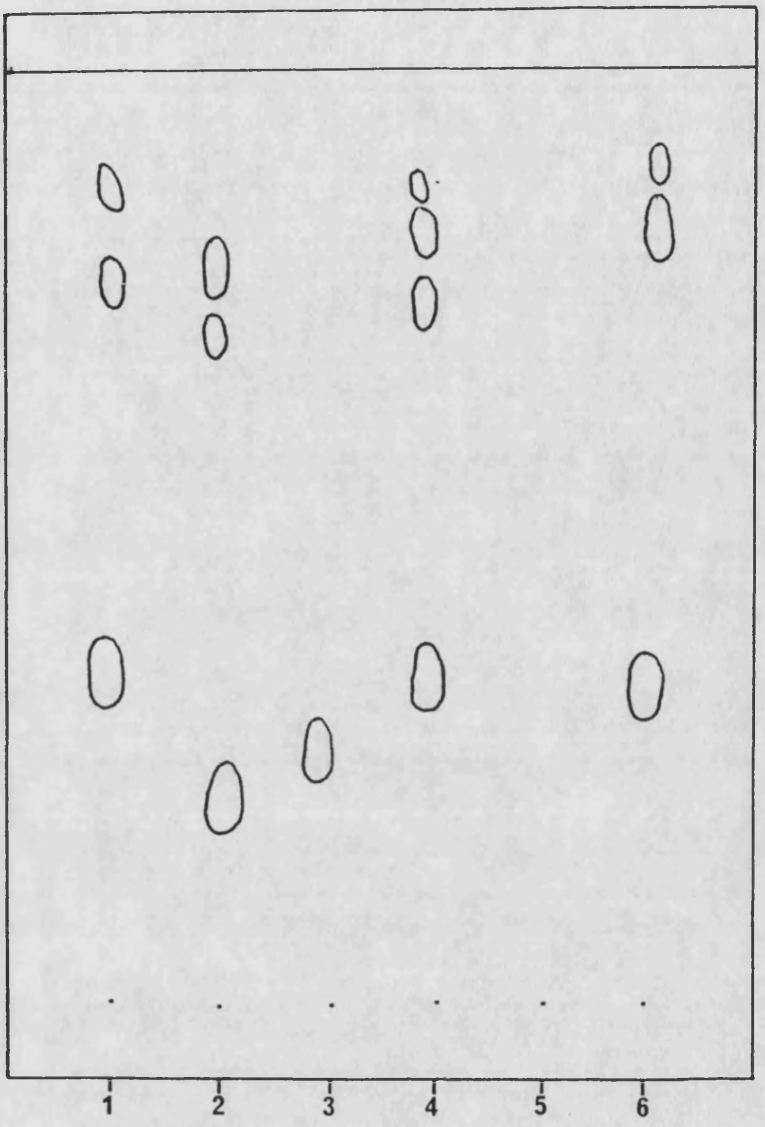
2.5 Discussion

Prior to detailed transformation studies on the dealkylation of codeine by *Candida tropicalis* strains, it was first necessary to establish the reliability of the technique used for assessing growth characteristics of the strains. Previous studies on the dealkylation of drugs have used filamentous fungi such as *Cunninghamella* sp. where microbial growth has been measured by dry cell weight (Sewell, 1982). However, this method is inconvenient, has limited accuracy and is not a direct measurement of viability of cells. Therefore, the surface spread method for counting viable cells was adopted for use in this study. The accuracy of the surface spread method for counting *Candida*



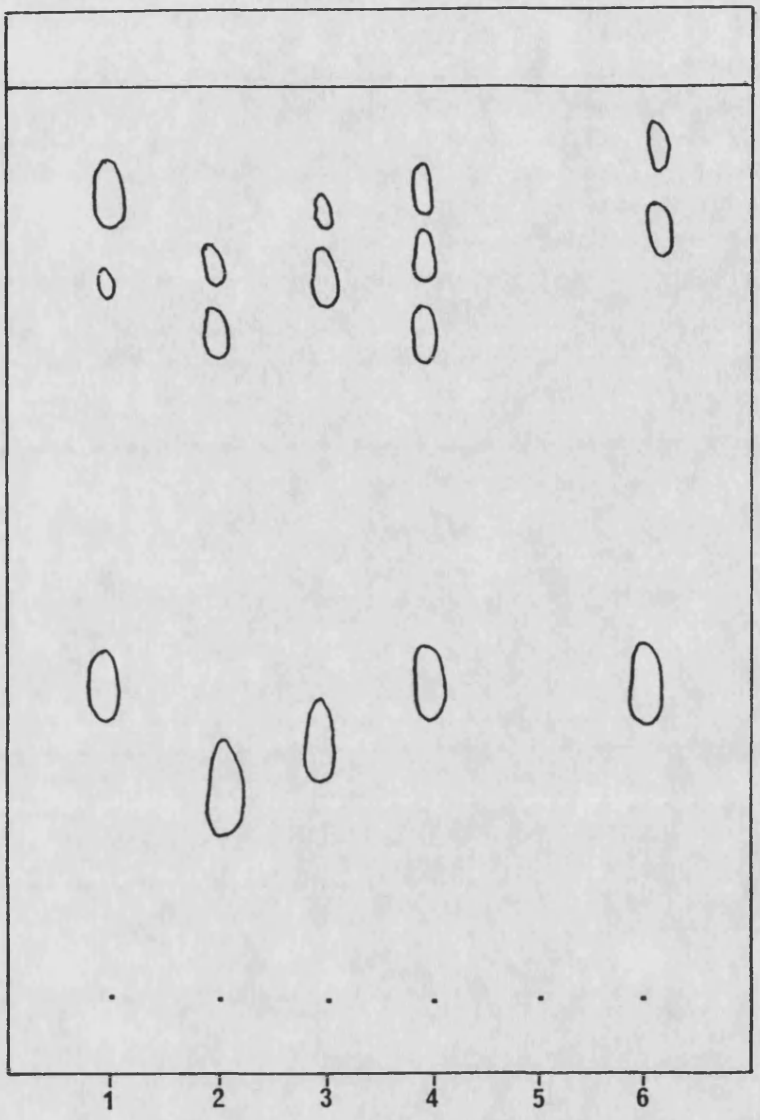
1. Codeine standard
2. Norcodeine standard
3. Morphine standard
4. Transformation extract from Candida tropicalis NCYC 997
5. Control: culture of Candida tropicalis NCYC 997 without substrate in chemically defined growth medium.
6. Control: sterile chemically defined growth medium containing 1mM codeine phosphate.

Figure 2·9 Thin layer chromatogram from culture of Candida tropicalis NCYC 997 grown with codeine (1mM) as sole carbon source.



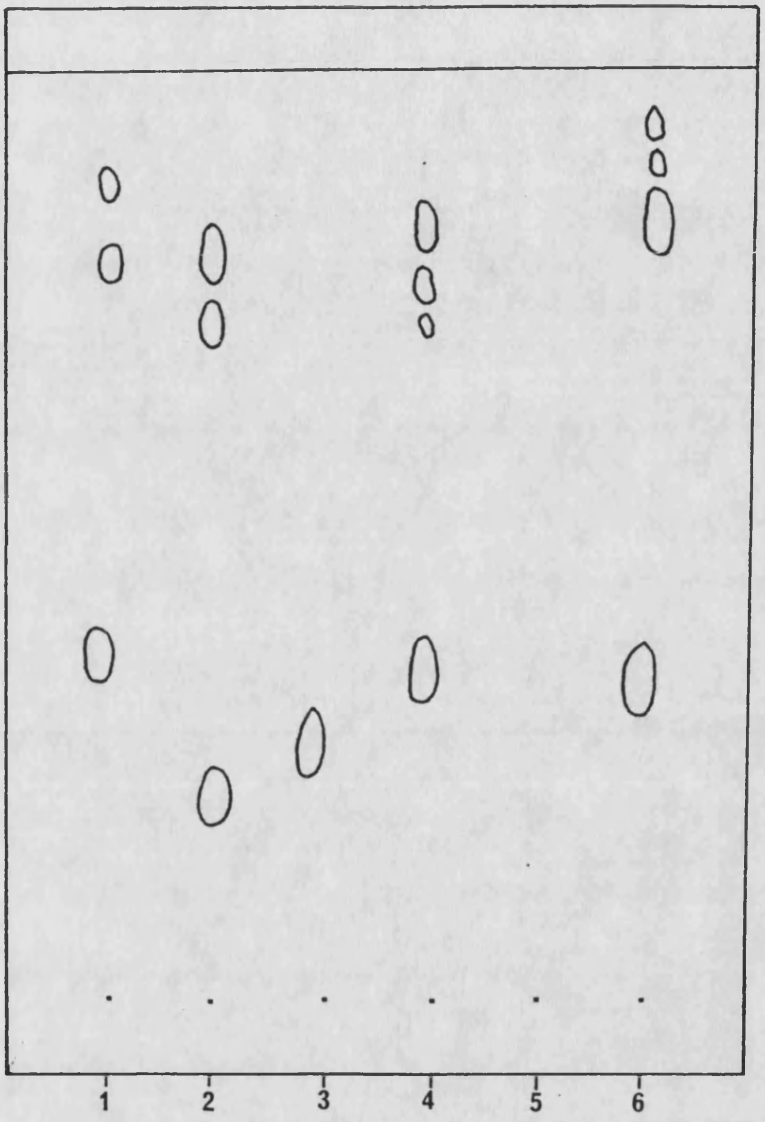
1. Codeine standard
2. Norcodeine standard
3. Morphine standard
4. Transformation extract from Candida tropicalis ATCC 20221
5. Control: culture of Candida tropicalis ATCC 20221 without substrate in chemically defined growth medium.
6. Control: sterile chemically defined growth medium containing 1mM codeine phosphate.

Figure 2·10 Thin layer chromatogram from culture of Candida tropicalis ATCC 20221 grown with codeine(1mM) as sole carbon source.



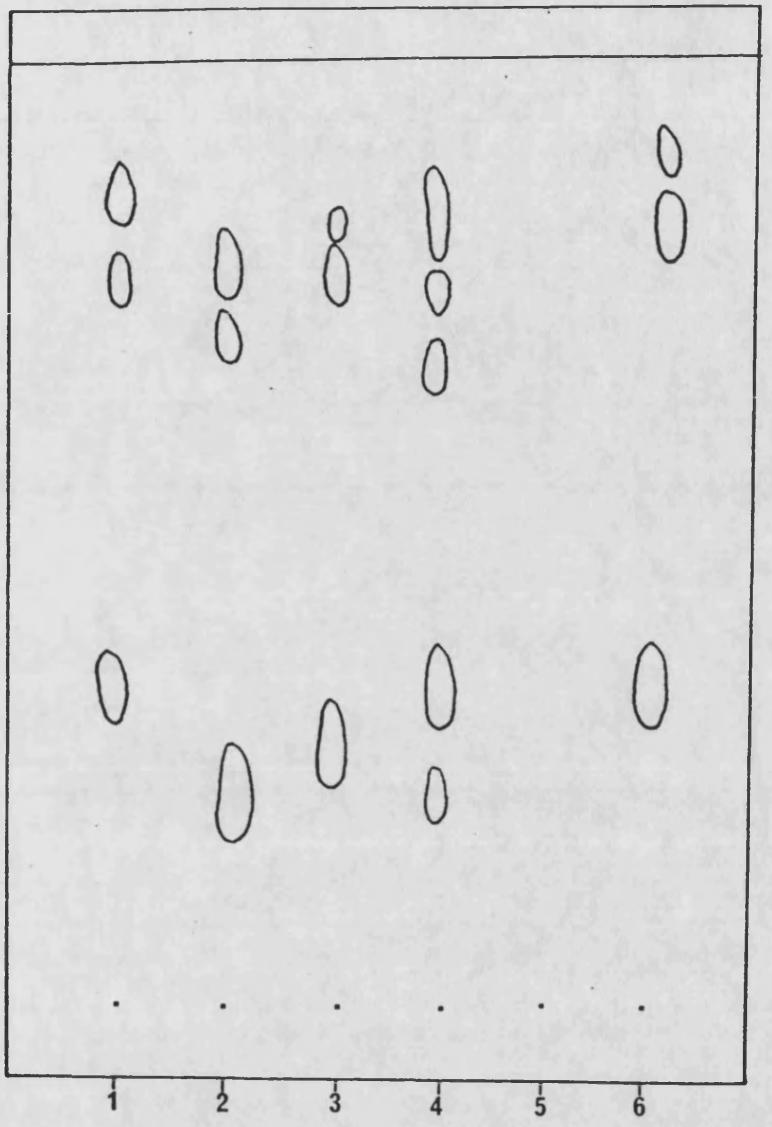
1. Codeine standard
2. Norcodeine standard
3. Morphine standard
4. Transformation extract of Candida tropicalis ATCC 20336
5. Control: culture of Candida tropicalis ATCC 20336 without substrate in chemically defined growth medium.
6. Control: sterile chemically defined growth medium containing 1mM codeine phosphate.

Figure 2·11 Thin layer chromatogram from culture of Candida tropicalis ATCC 20336 grown with codeine(1mM) as sole carbon source.



1. Codeine standard
2. Norcodeine standard
3. Morphine standard
4. Transformation extract from Candida tropicalis ATCC 22577
5. Control: culture of Candida tropicalis ATCC 22577 without substrate in chemically defined growth medium.
6. Control: sterile chemically defined growth medium containing 1mM codeine phosphate.

Figure 2·12 Thin layer chromatogram from culture of Candida tropicalis ATCC 22577 grown with codeine(1mM) as sole carbon source.



1. Codeine standard
2. Norcodeine standard
3. Morphine standard
4. Transformation extract from Candida tropicalis ATCC 32113
5. Control: culture of Candida tropicalis ATCC 32113 without substrate in chemically defined growth medium.
6. Control: sterile chemically defined growth medium containing 1mM codeine phosphate.

Figure 2·13 Thin layer chromatogram from culture of Candida tropicalis ATCC 32113 grown with codeine(1mM) as sole carbon source.

Sample	R_f Values of major spots
Codeine standard	30·8 - 37·3
Norcodeine standard	18·1 - 28·6
Morphine standard	24·1 - 33·1
Candida tropicalis NCYC 997	31·3 - 38·1
Candida tropicalis ATCC 20221	32·1 - 38·8
Candida tropicalis ATCC 20336	30·8 - 39·1
Candida tropicalis ATCC 22577	32·8 - 38·8
Candida tropicalis ATCC 32113	30·6 - 39·6
	20·9 - 23·2

Table 2·5 TLC migration data (R_f values) for standard solutions and extracts of cultures of Candida tropicalis strains grown using codeine as a sole carbon source in chemically defined growth medium.

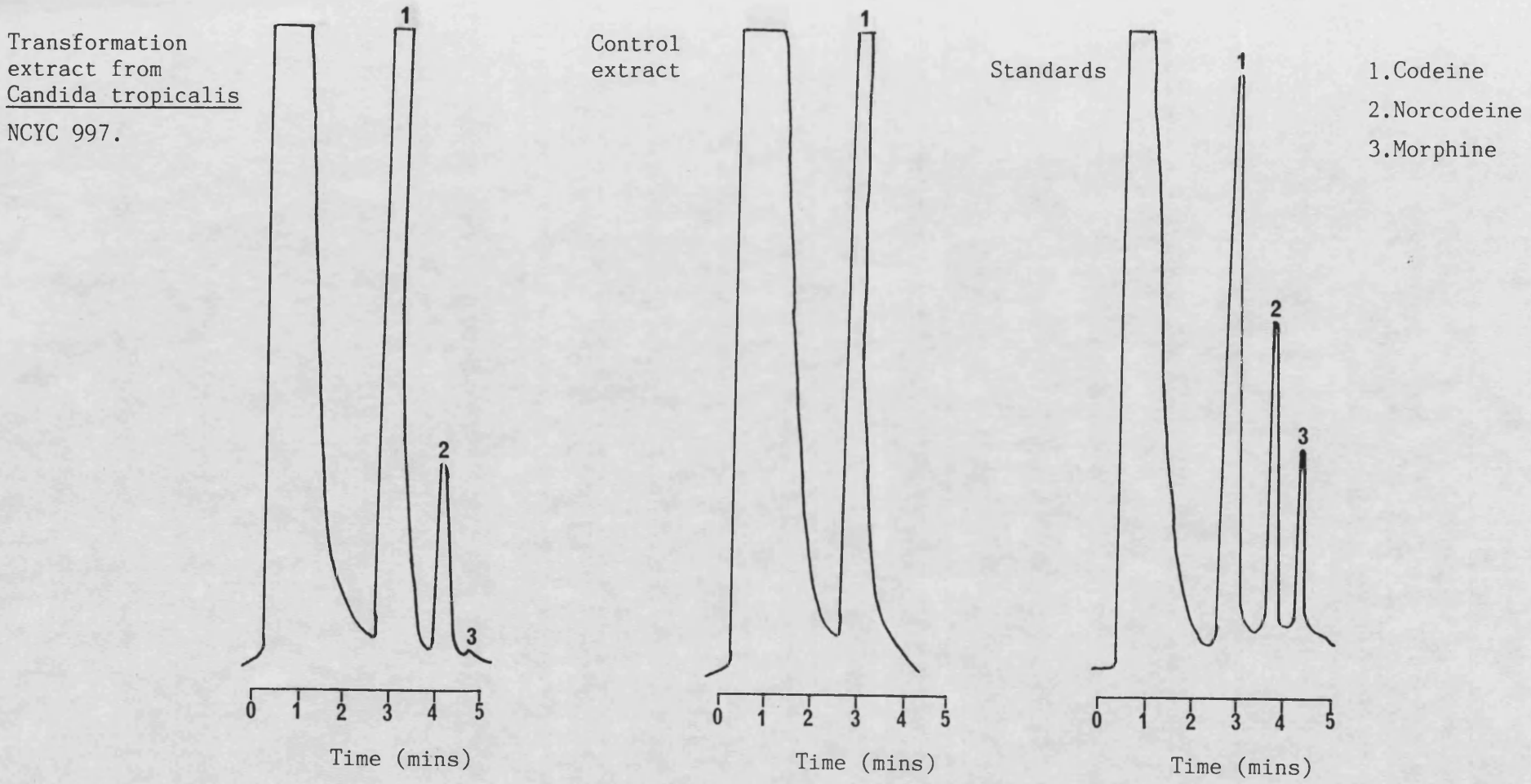


Figure 2·14 Typical GLC chromatograms from the analysis of extracts from cultures of Candida tropicalis NCYC 997 screened with codeine(1mM)

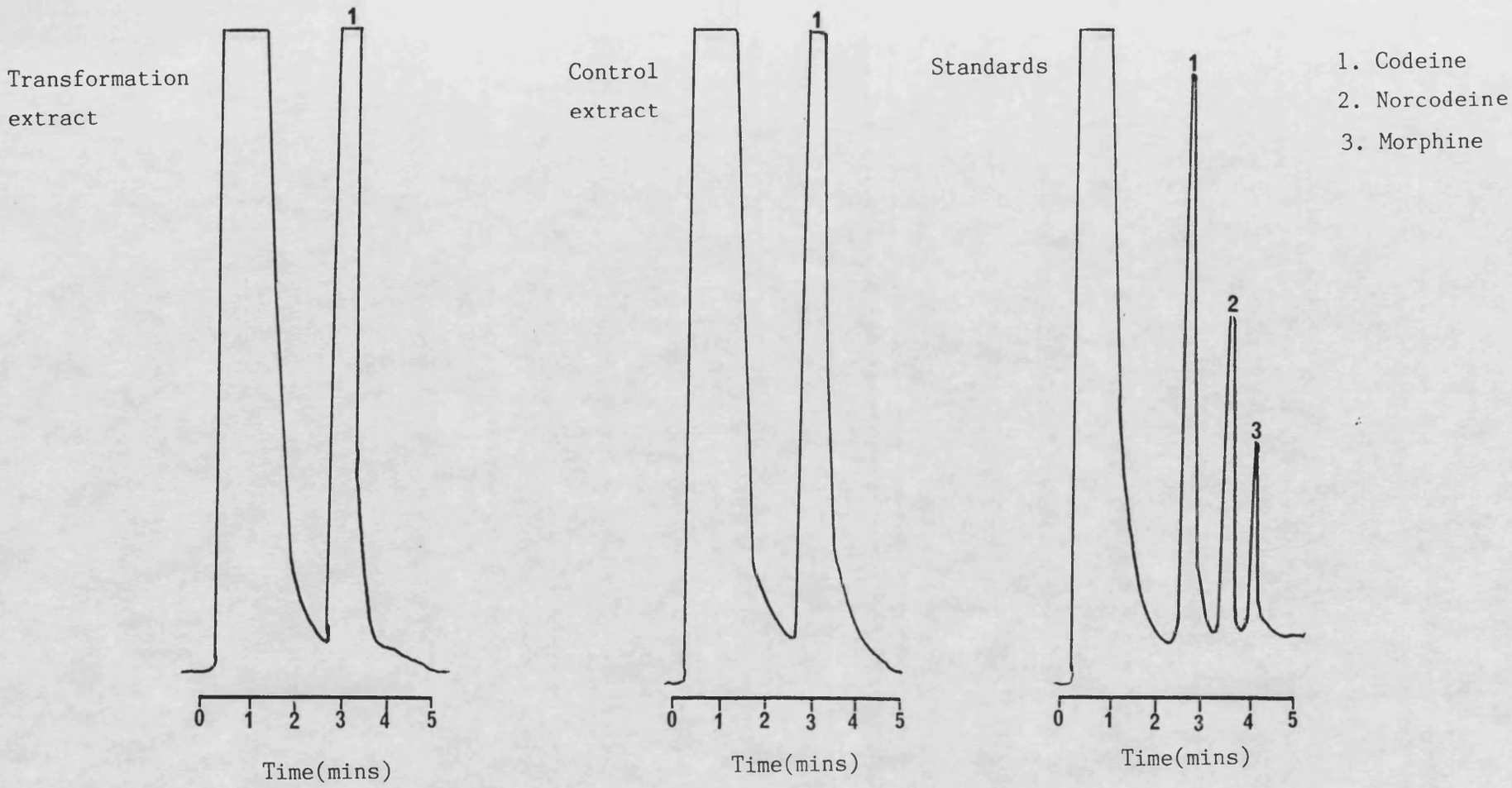


Figure 2·15 Typical GLC chromatograms from the analysis of extractsfrom cultures of *Candida tropicalis* ATCC 20221 screened with codeine(1mM)

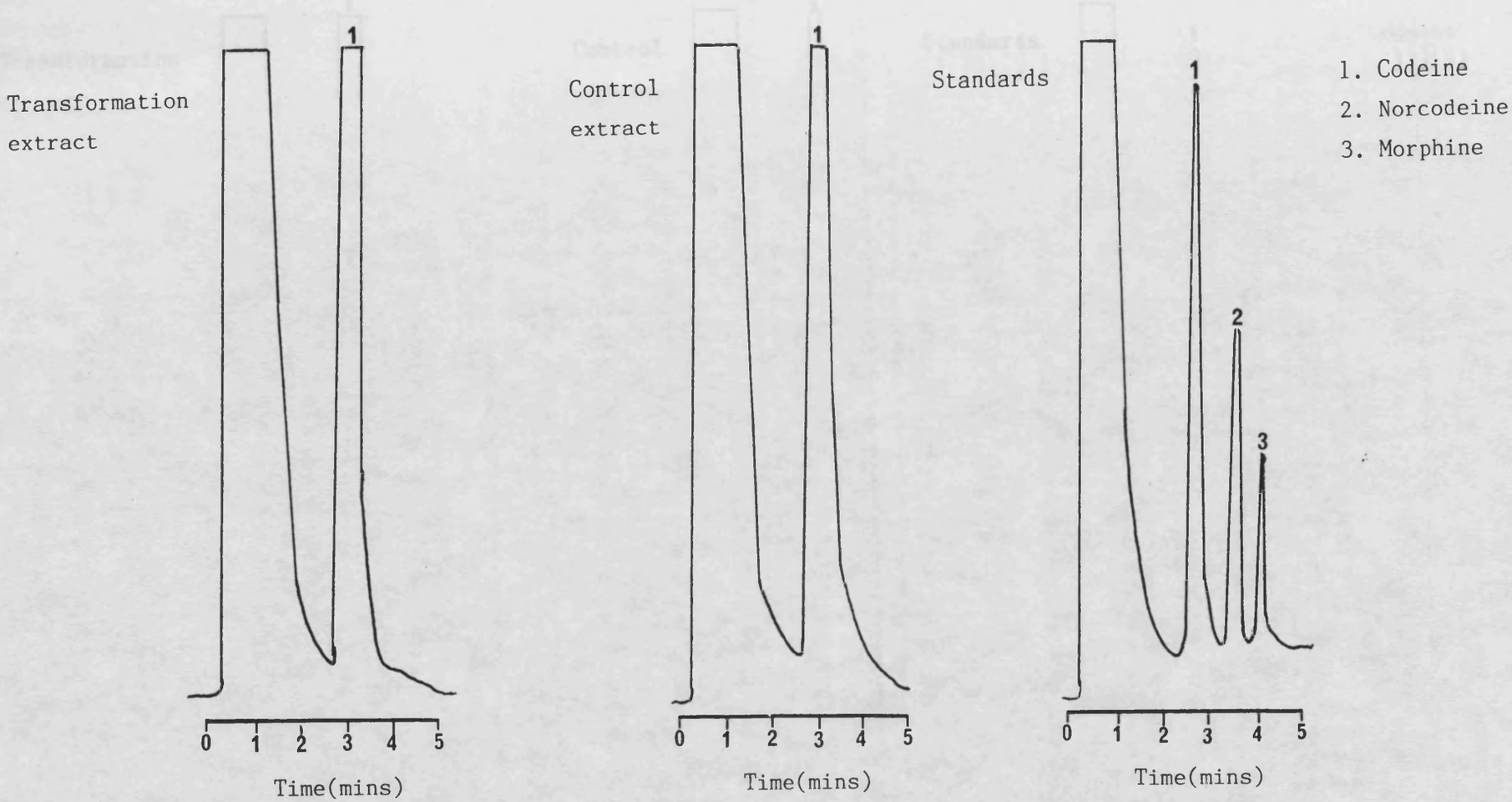


Figure 2·16 Typical GLC chromatograms from the analysis of extracts from cultures of *Candida tropicalis* ATCC 20336 screened with codeine(1mM)

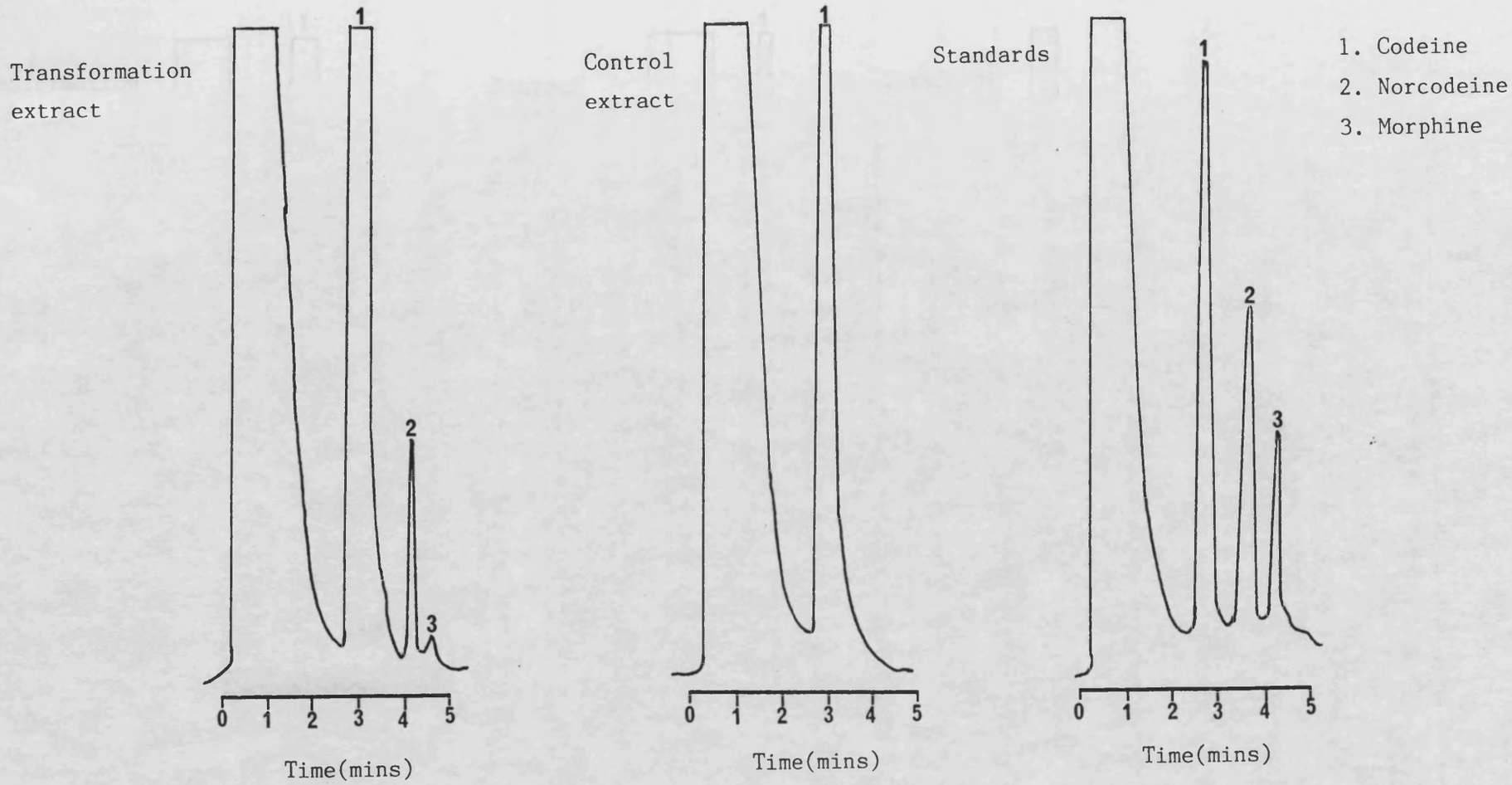


Figure 2·17 Typical GLC chromatograms from the analysis of extracts from cultures of Candida tropicalis ATCC 22577 screened with codeine(1mM)

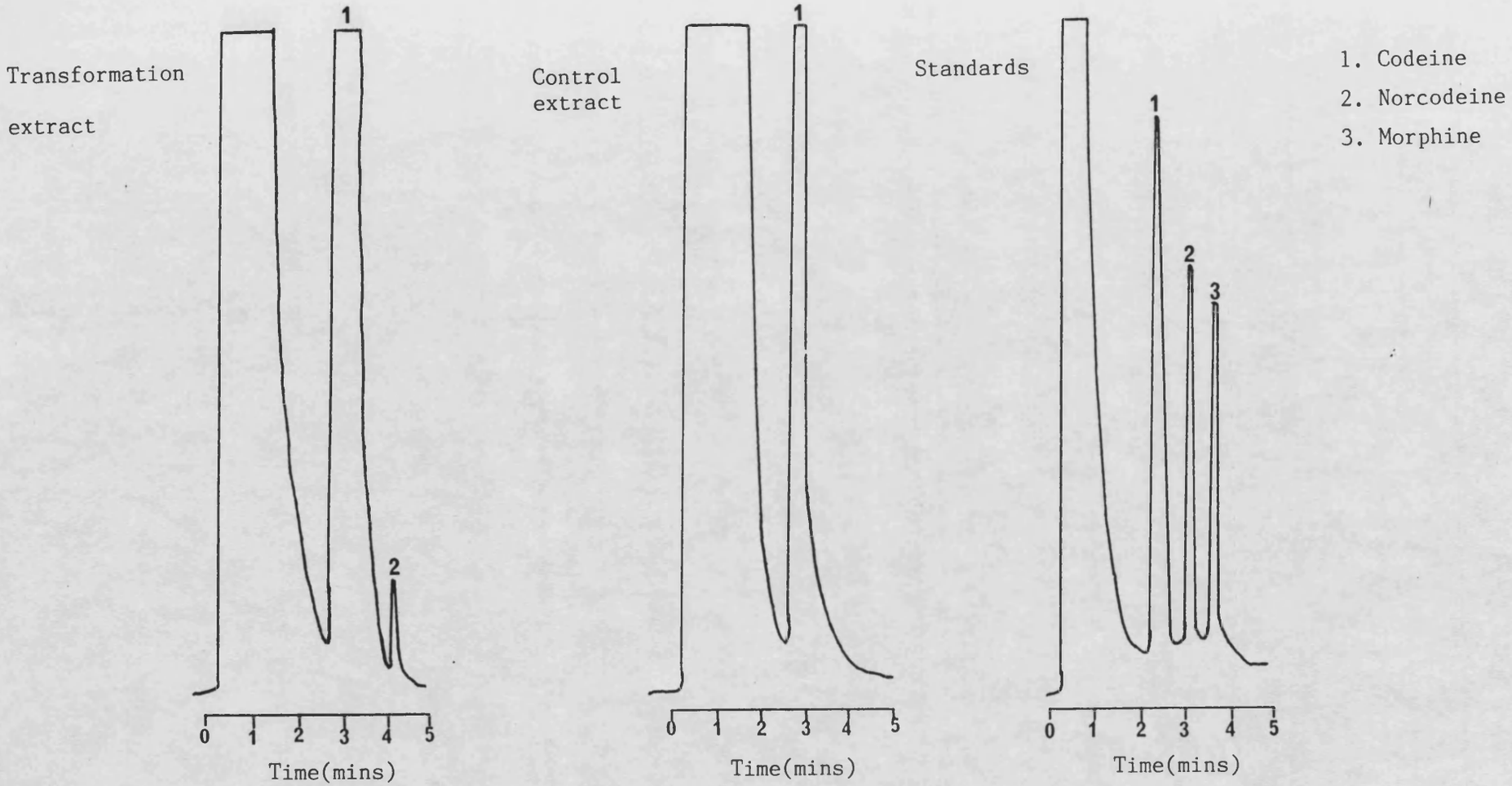


Figure 2·18 Typical GLC chromatograms from the analysis of extracts from cultures of Candida tropicalis ATCC 32113 screened with codeine(1mM).

Sample	Peak No.		
	1	2	3
Codeine standard	1·00		
Norcodeine standard		2·12	
Morphine standard			3·20
Control extracts	1·00		
Candida tropicalis NCYC 997	1·00	2·10	3·18
Candida tropicalis ATCC 20221	1·00		
Candida tropicalis ATCC 20336	1·00		
Candida tropicalis ATCC 22577	1·00	2·11	3·22
Candida tropicalis ATCC 32113	1·00	2·13	

Table 2·6 Retention data from GLC analysis of extracts from cultures of *Candida tropicalis* grown using codeine as a sole carbon source in chemically defined growth medium.

tropicalis is shown in Table 2.1. A variance ratio of 1.5 was obtained which was less than the tabulated value at the 5% probability level (3.01). This indicated that the error due to differences between samples was not significantly greater than that due to differences within samples. Statistically,therefore the surface spread method was suitable for measurement of viable cell counts. Theoretically, problems may have occurred with this method when used to count budding yeast such as *Candida tropicalis*. The budding yeast although potentially two viable cells, would only give rise to a single colony on the solid agar medium. The final count would then need to be expressed as colony forming units per ml. In practice, however,microscopic examination of the culture confirmed that the daughter cells separated rapidly from the parent cell and did not cause counting problems.

The surface spread method of counting although accurate was time consuming. Therefore measurement of the number of microorganisms in a culture by assessing the optical density at 550nm was investigated. Optical density measurements to assess the number of organisms has some limitations: the increase in size of spherical organisms during the growth cycle, would change the absorbance of the suspension. The change in absorbance is proportional to the four thirds power of the volume of the cell (Kavanagh *et al* 1972). Therefore the absorbance of a culture gives a more accurate measurement of total mass or volume rather than concentration of microorganisms.The calibration plot for *Candida tropicalis* ATCC 32113 of viable count against optical density at 550nm produced a linear relationship, with a correlation coefficient of 0.982 (Figure 2.3) This suggested that optical density

measurements between 0.1 and 0.6 could be used as a means of rapidly determining the approximate viable count of a culture. This may explain the differences observed in the shapes of the growth curves for *Candida tropicalis* ATCC 32113 plotted using viable count and optical density at 550nm (Figure 2.2). Optical density measurement was therefore considered an inaccurate assessment of number of viable cells because killed microorganisms would also absorb light. This may account for the absence of a stationary phase on the optical density plot (Figure 2.2).

In previous transformation studies it had been necessary to first grow the microorganism to the stationary phase using glucose, sucrose or succinate as the carbon source. On depletion of the initial carbon source, the transformation substrate was then added for study (Sewell, 1982). This prolonged the experimental procedure for days. In this study it was found that using a chemically defined growth medium for transformation studies, codeine was utilized as the sole carbon source by three of the *Candida tropicalis* strains tested, but different levels of growth were obtained. Comparison of growth on glucose with growth on codeine (Figures 2.4-2.8) showed significant differences. Generally the initial lag phase was highly pronounced using codeine. Although an exponential growth phase was present in all the growth curve plots, a stationary phase was more pronounced at a lower level of growth when using codeine as a sole carbon source. This may have been due to either a catabolic repression or catabolic inhibition effect of codeine. Okinaka and Dobrogosz (1967) have suggested that readily utilised carbon sources are the most effective in producing catabolic repression. The most

widely accepted mechanism for catabolic repression has been summarised by Demain *et al* (1979), involving the inhibition of adenylate cyclase by certain carbon sources. The transcription of an operon by RNA polymerase is initiated by the binding of a complex formed from cyclic adenosine 3' -5'-monophosphate (c-AMP) and a c-AMP receptor protein. Inhibition of adenylate cyclase by the catabolic products of a carbon source reduces the conversion of ATP to c-AMP. The low levels of c-AMP inhibit the transcription of operons which are subject to this control. Attempts to relieve catabolic repression in the presence of the carbon source by addition of c-AMP have met with limited success (Demain *et al*, 1979).

The screening experiments showed that codeine was demethylated by *Candida tropicalis* NCYC 997, ATCC 22577 and 32113. This was evident as characteristic norcodeine peaks appeared (RRT 1.51) on GLC analysis (Table 2.5). GLC analysis also provided evidence of some degree of O-demethylation by appearance of morphine peaks for *Candida tropicalis* ATCC 22577 and NCYC 997. However the peak heights for morphine were very small compared to the norcodeine peaks. The norcodeine peak for *Candidia tropicalis* ATCC 22577 was only obtained with the detector at high sensitivity setting. In contrast, the norcodeine peak for *Candida tropicalis* ATCC 32113 was obtained at a relatively low detector sensitivity setting. TLC analysis for *Candida tropicalis* ATCC 32113 does not rule out evidence of N- and O-demethylation of codeine because fo overlap of spots. However this was not observed by GLC which only detected norcodeine.

During TLC analysis multiple spots developed on the TLC plates

for each standard and the transformation mixtures. The multiple spots were seperated into two groups. Single large spots were observed near the sample application points and smaller irregular spots near the solvent front. On calculating the R_f values of the smaller irregular spots there was no consistant trend in values, but a consistant trend was observed for the larger spots. On this basis the small irregular spots were not taken into account when calculating R_f values. The smaller irregular spots may have originated from impurities within the drug samples or the solvent used for dissolving extracted samples. It may be possible to identify the smaller minor spots by preparitive TLC.

The advantages of GLC over TLC are due to the combination of sensitivity, efficiency, reliability and speed of analysis of the technique. This is evident when the retention mechanisims of the two techniques are examined. In TLC polar organic materials such as silica or alumina are often used and the mobile phase is usually an organic solvent mixture. The surface of the silica consists mainly of slightly acidic silanol groups which may be associated with water molecules via hydrogen bonding. Silica is heated to 150°C to drive off the associated water and increase surface activity. Retention and selectivity are the result of adsorption of the polar functional groups of the solute molecules directly onto the surfaces of the packing. The solute retention is proprotional to the relative number and type of polar functionalgroups in the solute.

In GLC the individual components of a sample seperate by differential partitioning as they move through a packed column. The

column packing material consists of a liquid phase adsorbed onto a solid support of controlled porosity and defined particle size. The choice of the liquid phase is very important. A variety of liquid stationary phases are commercially available in a range of polarites. In TLC the choice of stationary phases is very limited. In GLC therefore a stationary phase may be selected which suits best the sample components to be analysed.

The data obtained suggested that of all the strains screened, *Candida tropicalis* ATCC 32113 had the most favourable profile regarding codeine transformation activity. *Candida tropicalis* ATCC 32113 was therefore selected for further codeine transformation studies.

CHAPTER THREE

GROWTH MEDIA DEVELOPMENT AND CODEINE TRANSFORMATION OPTIMIZATION FOR *Candida tropicalis* ATCC 32113

CHAPTER 3. GROWTH MEDIA DEVELOPMENT AND CODEINE TANSFORMATION OPTIMISATION FOR *Candida tropicalis* ATCC 32113

3.1 Introduction

The studies in the previous section have demonstrated the ability of *Candida tropicalis* ATCC 32113 to N-demethylate codeine. This *Candida* strain was particularly suitable because it N-demethylated codeine to produce norcodeine only. O-demethylation was not observed because morphine was not detected in the transformation mixtures. *Candida tropicalis* ATCC 32113 was also easy to grow in culture flasks and readily utilized codeine as a sole carbon source at 1 mM concentrations. The medium for growth of *Candida* strains has usually included ammonium salts, phosphate salts, trace elements and yeast extract (Mauersberger *et al*, 1980). A medium used by Hug *et al* (1974) having these components has been successfully used for growth of yeasts with an n-alkane carbon source. This medium was adapted for use in this study where the effects of varying some components of the medium was investigated. Cytochrome P-450 monooxygenase enzymes have been implicated in the N-demethylation of drug molecules (Gibson, 1984), and the induction of P-450 in yeasts growing on n-alkanes as the sole carbon source has been demonstrated for *Candida tropicalis* (Gallo *et al*, 1971; Gilewicz *et al*, 1979) and *Candida guilliermondii* (Tittelbach *et al*, 1976; Muller *et al*, 1979). The effect of n-alkanes on the transformation activity of *Candida tropicalis* was therefore investigated. Although 1,2 dichloroethane has been used for the extraction of alkaloids from

aqueous transformation mixtures (Mule, 1964; Sewell, 1982), most recent studies by Kunz *et al*, 1985 have used chloroform as the organic extraction solvent. The efficiency of these extraction solvents regarding extraction of codeine and norcodeine from the aqueous transformation mixtures was also determined.

From the results obtained in Chapter 2, the sensitivity and resolution efficacy of the TLC method was considered to be very poor. However, the GLC method was considered very sensitive and flexible for detecting the codeine metabolites. The GLC method therefore was quantitatively developed to study the N-demethylation of codeine. The aim of the studies reported in this section was to develop a suitable growth medium and conditions to optimise the codeine N-demethylation activity of *Candida tropicalis* ATCC 32113.

3.2 Materials

3.2.1 Growth Media

3.2.1.1. Chemically defined growth media

All chemically defined growth media were prepared using reagents of SLR grade (Fisons Scientific Apparatus, Loughborough) or Analar grade (BDH, Poole).

i) Chemically defined growth medium A

This was the "basal" growth medium originally used by Sewell, (1982) for *Streptomycetes* and fungi and subsequently by Stavrianakis, (1986) for *Candida* strains.

Salts mixture: (g) K_2HPO_4 (3.327), $NaH_2PO_4.2H_2O$ (0.732),

$(NH_4)_2SO_4$ (0.500), $MgSO_4.7H_2O$ (0.290), Disodium EDTA (0.290). Dissolved and made up to 1000 ml with glass distilled water. A double strength solution was prepared and sterilized by autoclaving at 121°C for 20 minutes.

Trace elements mixture: (mg) $CaCl_2.2H_2O$ (33), $(NH_4)_6Mo_7O_{24}.4H_2O$ (93), $FeSO_4.7H_2O$ (3.5), $ZnSO_4.7H_2O$ (5.5), $MnSO_4.4H_2O$ (0.75), $CuSO_4.5H_2O$ (0.20), $Co(NO_3)_2.6H_2O$ (0.125), $Na_2B_4O_7.10H_2O$ (0.09). Dissolved and made up to 1000ml using glass distilled water. A concentrated solution (50 times concentration) of the above was prepared and sterilized by 0.2µ pore size membrane filtration.

Vitamins solution: (mg) Thiamine (60), Nicotinic Acid (600), Pyridoxine (40), Folic Acid (24), Biotin (4). All supplied by Sigma Chemical Co., London. Dissolved and made up to 1000ml in glass distilled water. The solution was then sterilized by membrane filtration and stored in plastic universal bottles at -20°C.

Casein hydrolysate solution: 25 g of vitamin free casein hydrolysate (Oxoid L41) was dissolved in 250 ml of glass distilled water by gentle heating and stirring and the solution sterilized by autoclaving at 121°C for 20 minutes.

The chemically defined growth medium A was then prepared aseptically as below:

Salts mixture	25.0 ml
Trace elements solution	1.0 ml
Casein hydrolysate	5.0 ml
Vitamins solution	0.1 ml
Sterile glass distilled water	13.0 ml

Volumes of 100ml of chemically defined growth medium A were prepared

for transformation experiments.

ii) Chemically defined growth medium B

This medium was adapted from that used by Hug *et al*, 1974 but at double concentration. All ingredients were of either Analar grade (BDH Ltd, Poole) or SLR grade (Fisons Scientific Apparatus, Loughborough).

(g): $(NH_4)_2SO_4$ (10.0), $(NH_4)_2HPO_4$ (3.2), KCl (1.4), $MgSO_4.7H_2O$ (0.7), $CaCl_2.3H_2O$ (0.64), $FeCl_3.6H_2O$ (0.022), $MnSO_4.2H_2O$ (0.016), $ZnSO_4.7H_2O$ (0.014), $CuSO_4.5H_2O$ (0.0016), m-Inositol (0.093), CaPantothenate (0.06), Thiamine HCl (0.0094), Pyridoxine HCl (0.0023), Biotin (0.0005), Yeast autolysate (3.0). The components were dissolved in 800ml of glass distilled water in the order given above. The yeast autolysate was dissolved in the solution by gentle heating and the medium was made up to 1000 ml with glass distilled water. The medium was then adjusted to pH 5.5 using dilute NaOH and sterilized by autoclaving at 121°C for 15 minutes.

3.2.1.2. Carbon Sources

Glucose and sodium succinate (Sigma Chemical Co., London) were dissolved in glass distilled water to produce 10% w/v solutions. Solutions were then sterilized by 0.2µ membrane filtration prior to use. Codeine phosphate was prepared as a 0.1M solution with respect to codeine by dissolving in glass distilled water. The solution was sterilized prior to use by 0.2µ membrane filtration.

3.3 Methods

3.3.1. Cell Counts and Growth Curves

It was established in Chapter 2 that *Candida tropicalis* ATCC 32113 grew as single cells without formation of filaments. Therefore growth of the microorganism was measured by the surface spread viable counting technique. This technique was established as accurate and reliable, but time consuming. Optical density measurements at 550 nm provided a rapid alternative for the assessment of the cell viability of culture inocula. For construction of growth curves, the surface spread technique was used as in Section 2.3.2 and 2.3.4.

3.3.2. Extraction Procedures

The following procedure was used for extracting codeine and norcodeine from chemically defined growth media. Microbial growth was first removed from the contents of each flask by centrifugation (3500rpm, 5 mins). Chloroform (HPLC grade) 50 ml, was added to the supernatant contained in a separating funnel. The mixture was then gently swirled and the aqueous phase basified to pH 11-11.5 by dropwise addition of dilute sodium hydroxide solution. The mixture was again swirled gently for 2-3 minutes and the phases allowed to separate. The lower organic phase was drawn off and retained. The aqueous phase was further extracted with HPLC grade chloroform (2 x 50 ml). The organic phases were combined and dried using anhydrous $MgSO_4$ and, after filtration, evaporated to dryness using a rotary evaporator. The residue was then reconstituted into THF (2.0 ml) and

used for analysis by GLC.

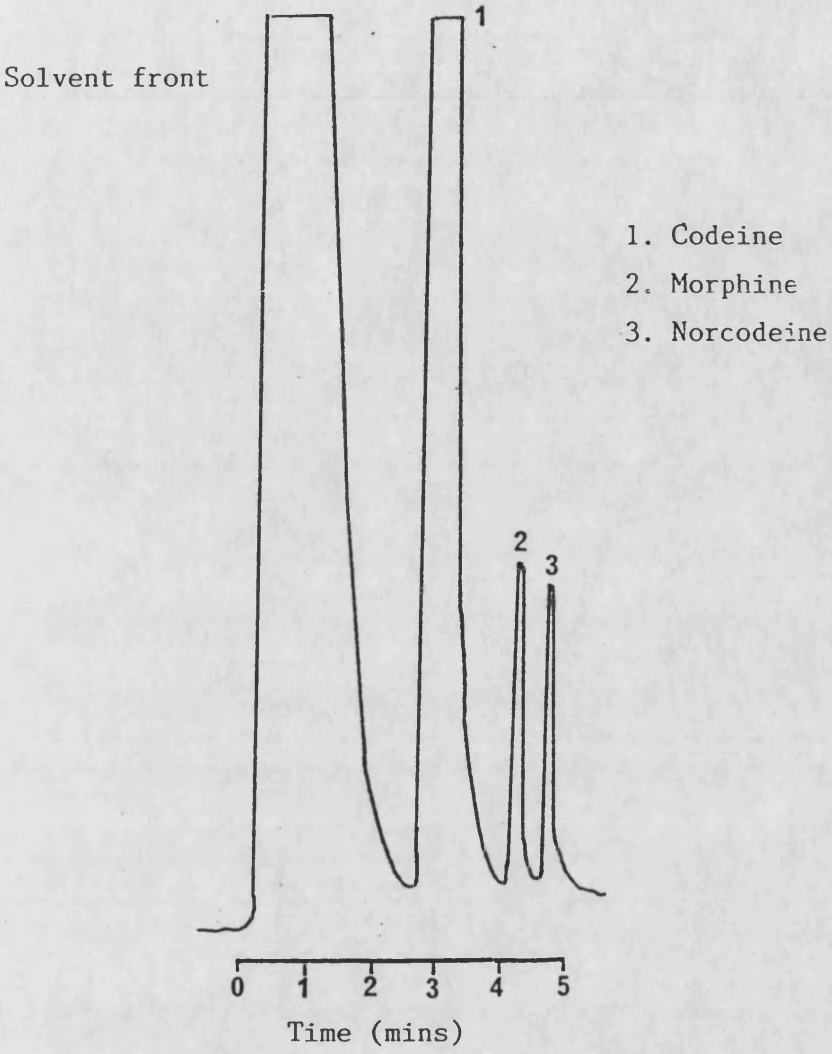
3.3.3. Quantitative GLC Analysis

GLC is an analytical technique that was extensively used in this study. The general principles of GLC have been well documented (Bristow and Knox, 1977) The qualitative GLC method used in Chapter 2 had established sufficient resolution of codeine and norcodeine, and derivatisation of the samples was found to be unnecessary using the 3% OV-17 stationary phase. The OV-17 stationary phase used at 250°C and nitrogen carrier gas flow of 120 ml min^{-1} produced a satisfactory chromatogram characterised by a short analytical time of less than 5 minutes, symmetrical peak shapes and peak heights greater than 10mm (Figure 3.1). Injector and detector temperatures were maintained at 270 and 300°C respectively in all experiments.

To compensate for minor fluctuations in sample volumes injected, variable column behaviour and gas flow, a known amount of an internal standard was added to the sample. A peak height ratio was calculated from the peak height (mm) of the analyte of interest and peak height (mm) of the internal standard.

$$\text{Peak Height Ratio} = \frac{\text{Height of analyte peak (mm)}}{\text{Height of internal standard peak (mm)}}$$

Morphine was chosen as the internal standard in the qualitative studies in Chapter 2. Morphine was also found to be a suitable internal standard for the quantitative studies and fulfilled all the criteria of an internal standard (Pattison, 1978).



Analytical conditions

Column: 4 metre glass 4mm i.d. packed with 3% OV-17 on chromosorb WHP 80/100 mesh

Oven temperature: 250°C

Carrier gas: nitrogen

Detector range: 1×10^3

Figure 3·1 Typical GLC chromatogram for the standard solutions at the conditions stated. Chromatogram characterised by short analysis time, symmetrical peak shapes and peak heights greater than 10mm.

3.3.1. Calibration Procedures

A calibration procedure was established as follows; a THF solution (1.0 ml) was prepared containing codeine (20 mg) and norcodeine (10 mg). Sample volumes ranging from 100 µl to 5 µl were then transferred into glass vials using a 100 µl syringe. The concentrations of codeine and norcodeine in each sample are shown in Table 3.1

Sample No.	Volume (µl)	Equivalent Concentration mg/ml	
		Codeine	Norcodeine
1	100	20.0	10.0
2	75	15.0	7.5
3	50	10.0	5.0
4	25	5.0	2.5
5	10	2.0	1.0
6	5	1.0	0.5

Table 3.1 Calibration samples prepared for GLC assay of codeine and norcodeine

A solution of morphine sulphate (1 $mgml^{-1}$) in THF (100 µl) was added to each calibration sample as the internal standard. Volumes (1 µl) were then assayed in duplicate. Plots of peak height ratios

against concentration for codeine and norcodeine are shown in Figure 3.2. Each plot was subjected to regression analysis and the data are shown in Figure 3.2. The content of codeine or norcodeine in a sample may be determined by dividing peak height ratio by the slope of the appropriate calibration curve. Between assays, the GLC calibration was occasionally checked by injecting a standard solution of codeine and norcodeine. The instrument was re-calibrated if the peak height ratio deviated more than 5% from the calibration value.

The efficiency of a column is given by the number of theoretical plates (N) in a column where

$$N = 16 \frac{x^2}{y}$$

x = retention time of a peak

y = width (mm) of given peak at base.

1200 or more theoretical plates per column were required for pharmacopoeial GLC assays (British Pharmacopoeia,1982). Using the norcodeine peaks (RRT 3.68) the value of N for the OV-17 column was calculated as:

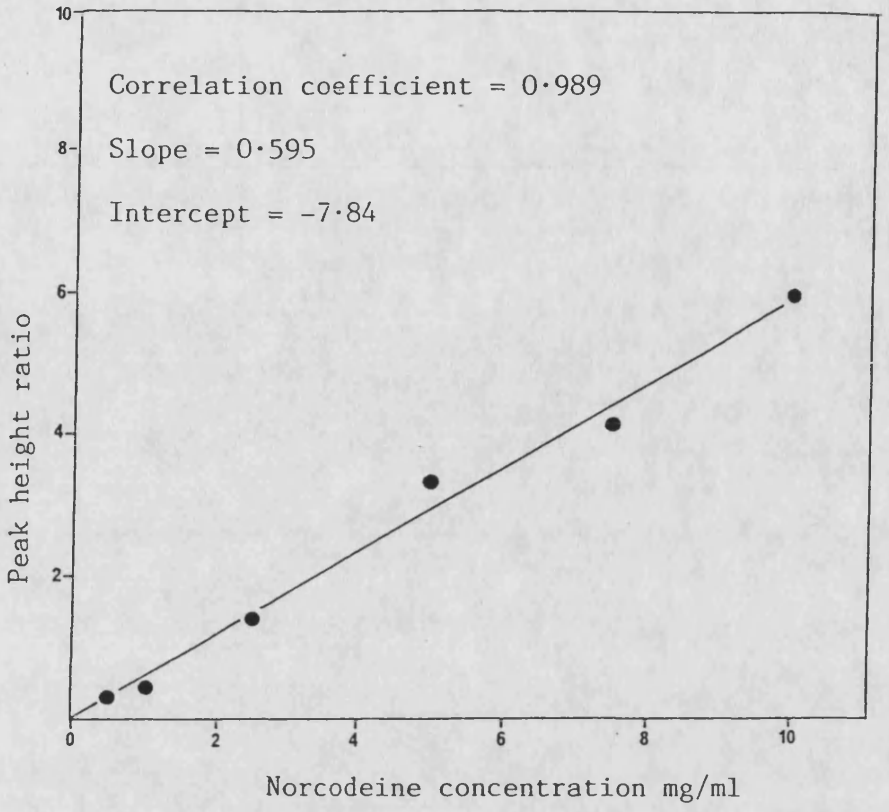
$$N = 16 \frac{3.68 \times 60}{3.2} = 76,176$$

If the efficiency of the column fell to a value below 20000, the column was repacked.

3.4. Experimental

3.4.1. Efficiency of Extraction Procedures

For accurate determination of codeine transformation, it was



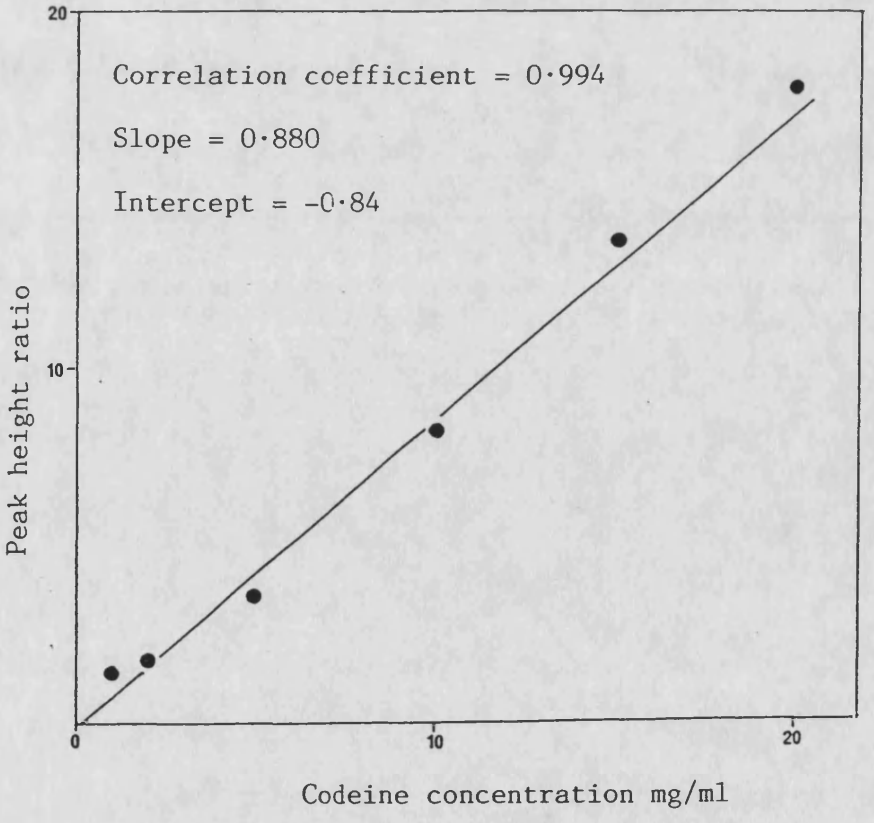


Figure 3·2 GLC calibration plots for codeine and norcodeine

necessary to establish the efficiency of extraction of codeine and norcodeine from chemically defined growth media.

Standard solutions of codeine phosphate and norcodeine hydrochloride in glass distilled water were prepared. Volumes (2ml) of each solution were added to each of five flasks containing 100 ml chemically defined growth medium B, as in Table 3.2.

Flask No.	Final Concentration (mM) in 100ml	
	Codeine	Norcodeine
1	1.0	0.1
2	0.8	0.08
3	0.6	0.06
4	0.4	0.04
5(control)	0	0

Table 3.2 Final base concentrations of codeine and norcodeine in each flask of chemically defined growth medium B (100ml)

The contents of each flask were extracted using the procedure described in Section 3.3.2. The organic solvents used in the assesment of extraction efficiency were chloroform (HPLC grade) and 1,2 dichloroethane containing 10% 2-methylpropanol. The total amounts of codeine and norcodeine recovered from each flask using

each organic solvent were plotted against the amounts of codeine and norcodeine added to each flask (Figures 3.3 and 3.4).

Extraction efficiencies of 90% and 92% for norcodeine and codeine respectively, using chloroform, were obtained. The extraction efficiencies were independent of the concentration of the compounds over the range used. 1,2-dichloroethane with 10% 2-methylpropanol proved a poor organic solvent for extraction purposes. Recoveries of only 24% and 15% for codeine and norcodeine respectively were obtained.

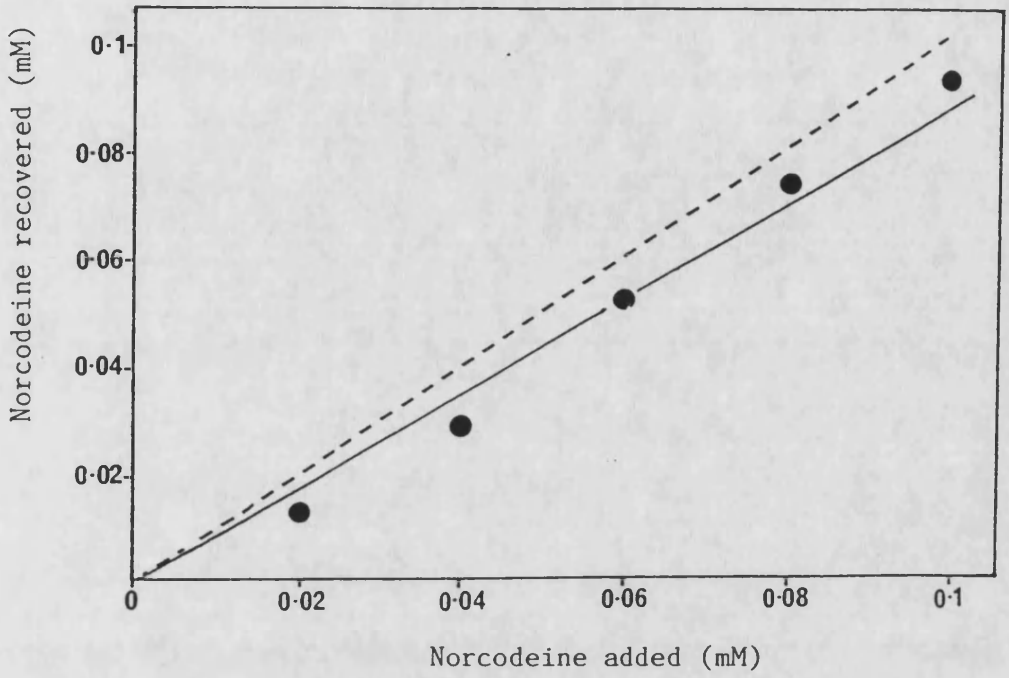
It was therefore concluded that HPLC grade chloroform was suitable for extraction of both compounds from medium B in all subsequent experiments.

3.4.2. Measurement of Codeine Transformation

It was assumed that all the norcodeine in transformation cultures arose from codeine transformation by cells. Duplicate injections were made and codeine and norcodeine peak heights (mm) were divided by internal standard peak heights. Mean peak height values were calculated for each compound and divided by the value for the slope of the respective calibration curve. This provided the weight (mg) of codeine and norcodeine recovered from the culture. All weights were converted to molar quantities and the percentage molar conversion yield in a culture mixture was calculated as:

$$\% \text{ molar conversion yield} = \frac{\text{Amount of norcodeine recovered (mM)}}{\text{Amount of codeine added (mM)}}$$

where 90% recovery of norcodeine was assumed.



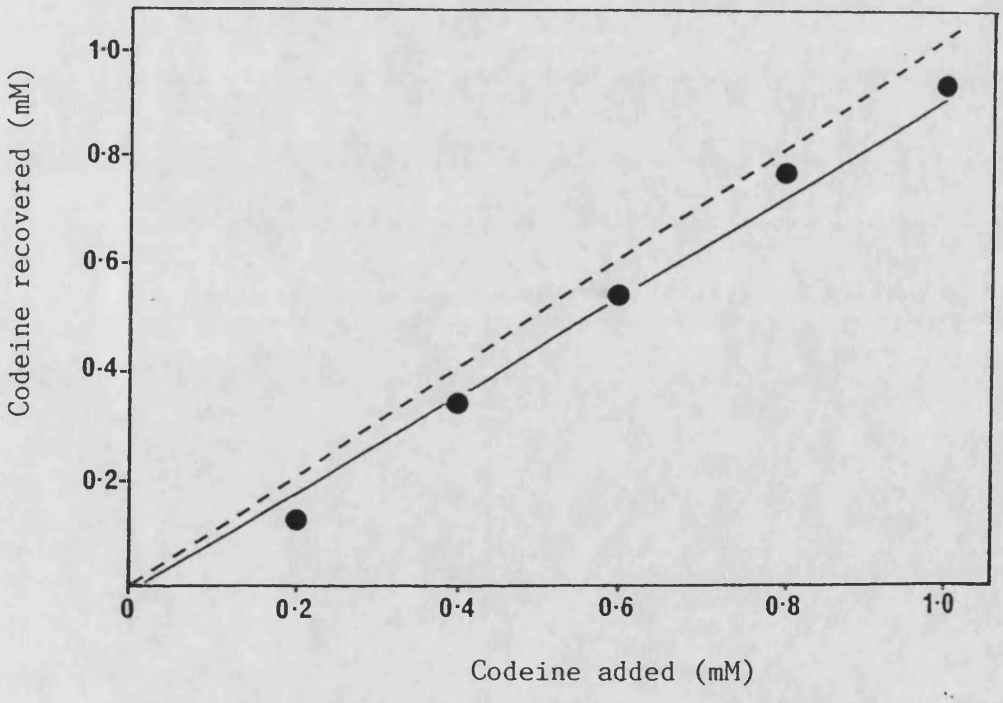
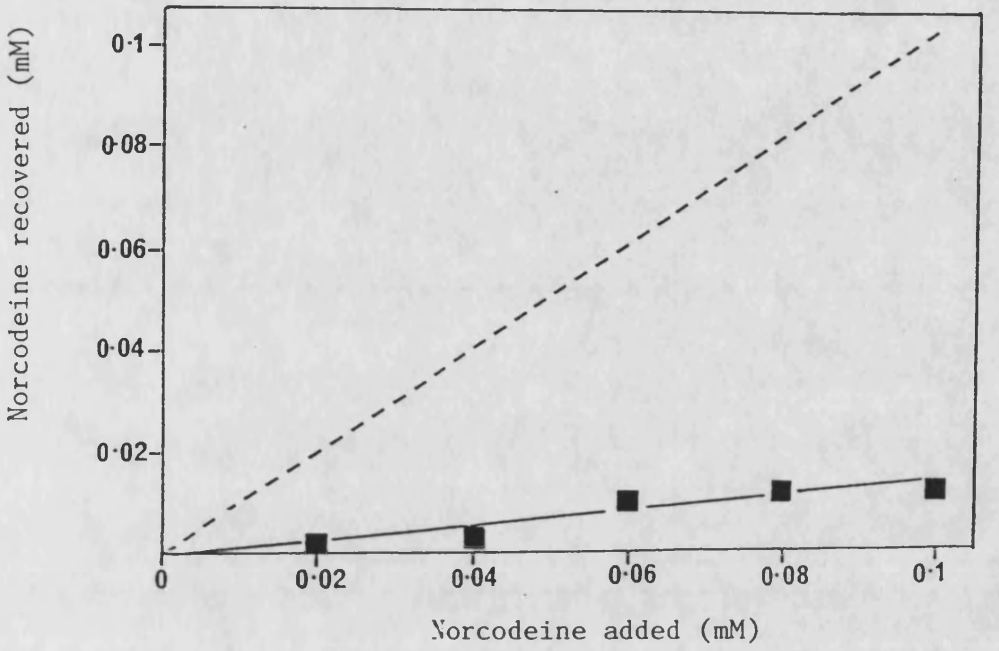


Figure 3·3 Recoveries of codeine and norcodeine from chemically defined medium B after 3 extractions using HPLC grade chloroform as solvent. Dotted line represents 100% recovery.



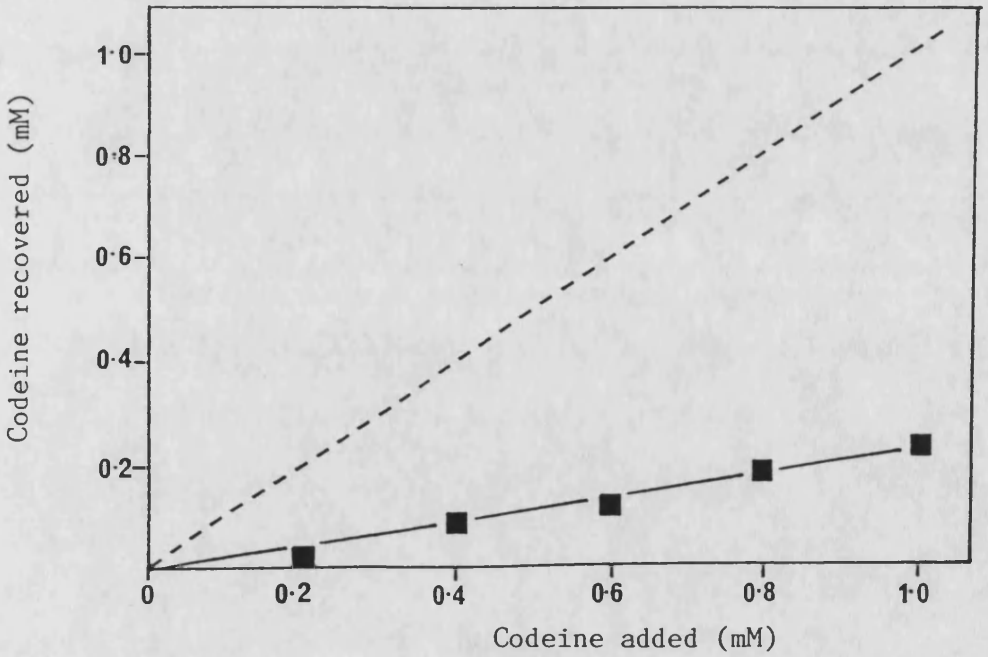


Figure 3·4 Recoveries of codeine and norcodeine from chemically defined medium B after 3 extractions using HPLC grade 1,2 dichloroethane with 10% 2-methyl propanol as solvent. Dotted line represents 100% recovery.

Example calculation

For *Candida tropicalis* ATCC 32113 grown in chemically defined growth medium B containing 1.6 mM codeine final concentration. From the GLC analysis:

Injection	Peak height (mm)		Peak height ratio
	Norcodeine	Morphine	
1	148	87	1.70
2	152	87	1.75
			1.73(mean)

Value of slope of norcodeine calibration curve = 0.592

Therefore norcodeine recovered = $\frac{1.73}{0.592 \times 285}$ = 10.25μM

(MWt. 285)

% molar conversion yield = $\frac{0.01025 \times 100}{1.6}$ = 0.64%

3.4.3 Assessment of Growth in Different Media

The chemically defined growth medium A was based on a mineral salts and trace elements mixture containing vitamins and casein hydrolysate as a nitrogen source. The chemically defined growth medium B was based on the synthetic medium initially used by Hug *et*

al,(1974) for the growth of *Candida tropicalis* on hydrocarbon sources, with the concentration of each component doubled and yeast extract added as an extra nitrogen source. This type of chemically defined growth medium has been used by Gmunder *et al* (1981a) for the growth of *Candida tropicalis* in chemostat studies.

The carbon sources used for study were glucose and codeine phosphate. Glucose, as a carbon source, has been used extensively for yeast transformation studies (Yoshida and Aoyama, 1984). The glucose concentration was maintained at 1% w/v and codeine concentration at 1.6 mM.

A primary culture of *Candida tropicalis* ATCC 32113 was set up. After 18 hours incubation, the cells were removed by centrifugation and resuspended in an appropriate prewarmed chemically defined growth medium . 10 ml volumes were used for inoculation of secondary cultures. Four secondary cultures of each chemically defined growth medium were set up for each carbon source used, and incubated for a maximum of 24 hours. Samples from each flask were taken at 4 hour intervals during incubation and subjected to viable count determination as described in Section 2.3.2. The viable count data was used to construct growth curves. The growth curves plotted for medium A and B using each carbon source are shown in Figures 3.5 and 3.6. The pH of the cultures was also determined during incubation. The pH profiles are shown with the growth curves in Figures 3.5 and 3.6.

The data showed that *Candida tropicalis* cells were able to grow readily in chemically defined medium B with either glucose or codeine

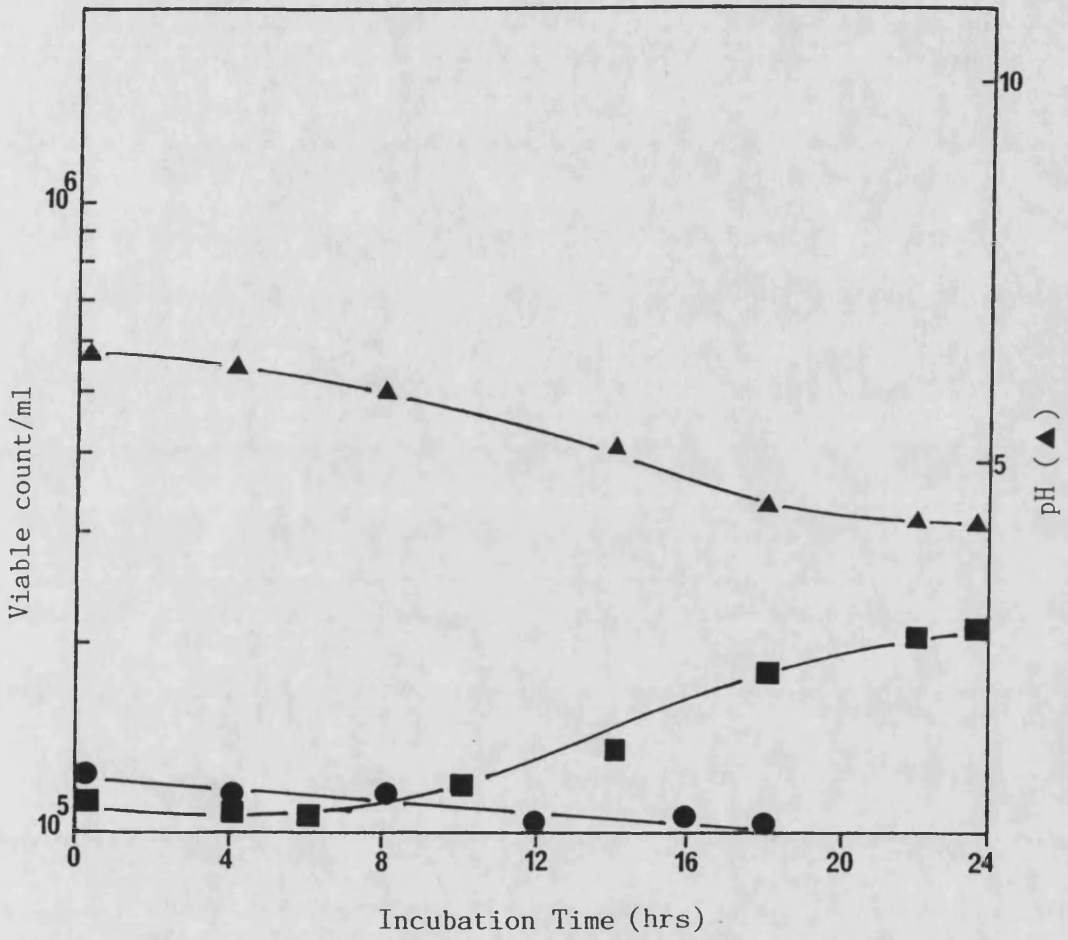


Figure 3·5 Growth curves for Candida tropicalis ATCC 32113 grown in chemically defined medium A with glucose (■), and codeine (●) each as sole carbon source . pH of both cultures during incubation (▲). Incubation temperature 30°C.

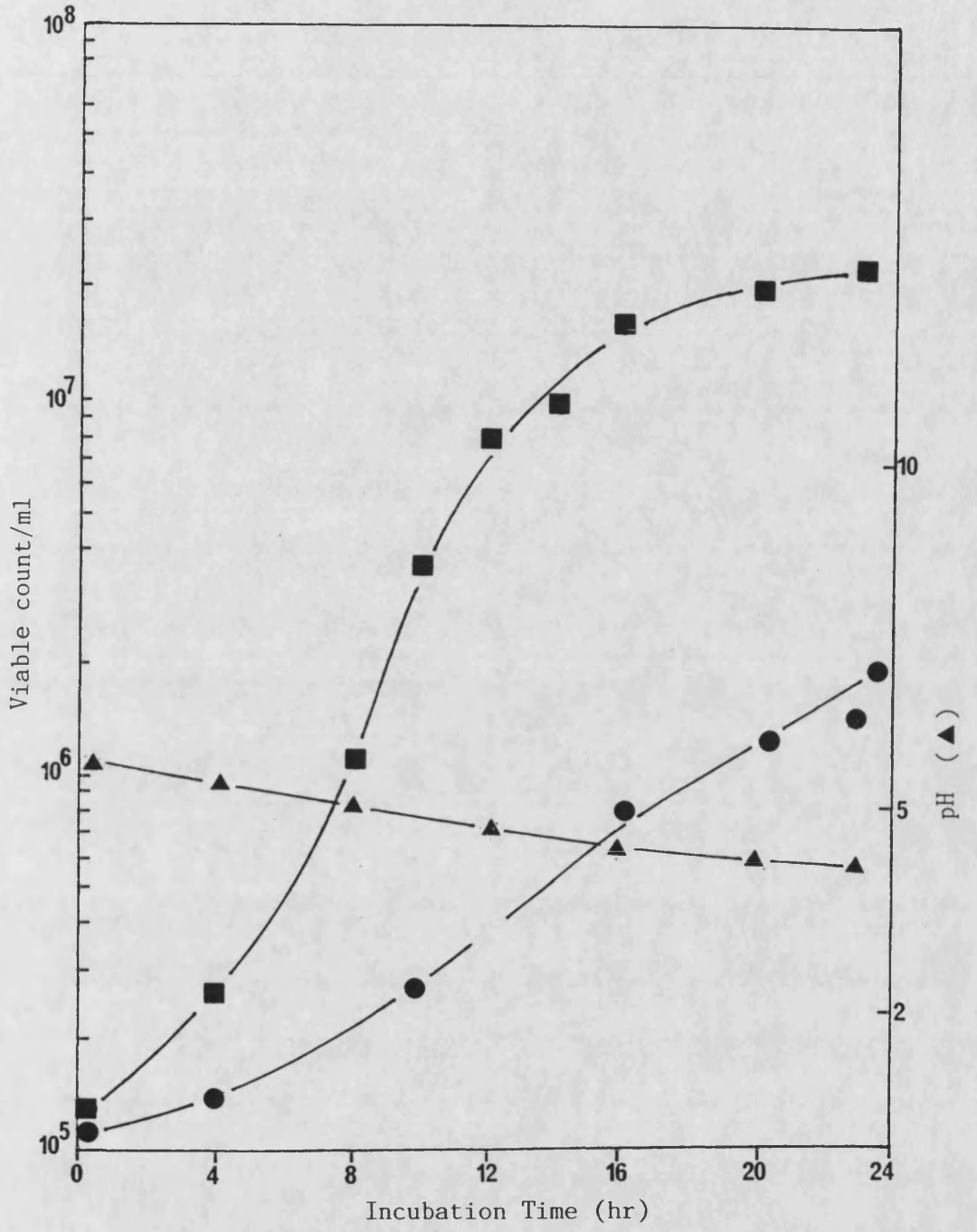


Figure 3·6 Growth curves for Candida tropicalis ATCC 32113 grown in chemically defined medium B with glucose (■) and codeine (●) each as sole carbon source . pH of both cultures during incubation (▲).
Incubation temperature 30°C.

as sole carbon sources (Figure 3.6). The rate of growth was greatest with glucose as the carbon source in medium B. With codeine as the carbon source in medium B, there was an increase in the initial lag phase of growth, and the viable count was lower than that with glucose at the end of the 24 hour incubation period. The pH of the culture was observed to decrease by 1 unit over the incubation period for both glucose and codeine carbon sources.

Candida tropicalis cells grown on glucose in medium A showed a very slow rate of growth during the incubation period (Figure 3.5). However the viable count was observed to decrease with codeine as the sole carbon source in medium A. The pH of cultures with glucose and codeine as carbon sources in medium A decreased by 2 units over the incubation period.

From the results obtained it was concluded that chemically defined medium B was the most suitable medium for subsequent studies. It was also shown that the *Candida tropicalis* ATCC 32113 cells were able to grow on codeine as a sole carbon source. Codeine at 1.6mM was therefore used as a sole carbon source in medium B for subsequent transformation studies.

3.4.4 Effect of n-alkane carbon sources on growth of primary cultures

Previous demethylation studies using yeasts have shown that

cytochrome P-450 monooxygenases may be responsible for catalysing the reaction (Aoyama *et al*, 1981a; 1984). These enzymes have been shown to be present in *Candida tropicalis* strains where their production is enhanced by growth on hydrocarbons (Duppel *et al*, 1973, Sanglard *et al*, 1986).

Prior to examining the effect of primary culture carbon sources on codeine transformation, it was necessary to investigate the effect of the alkanes on the growth characteristics of primary cultures. The n-alkanes examined were hexadecane, tetradecane and decane since these have been reported to induce cytochrome P-450 enzymes in *Candida tropicalis* strains (Duppel *et al*, 1973).

Two flasks were prepared for each of the alkanes in chemically defined medium B. The alkanes served as sole carbon sources and a concentration of 1%w/v was used for each. An 18 hour culture of *Candida tropicalis* ATCC 32113 in TSB was prepared as described in Section 2.3.1. The culture was centrifuged (3500rpm, 5 mins) and resuspended in chemically defined medium B as described in Section 2.3.1. Volumes (10ml) were used to inoculate each of the prewarmed alkane containing flasks and these were incubated with shaking (150 rpm) at 30 °C for 24 hours. Control flasks with codeine (1.6mM) as the sole carbon source in medium B were also inoculated and incubated under the same conditions.

Samples were taken from each of the alkane containing flasks and codeine containing flask every 4 hours during the incubation period. The samples were subjected to viable count determination as decribed in Section 2.3.2. The growth curves plotted for the alkane and codeine carbon sources are shown in Figure 3.7.

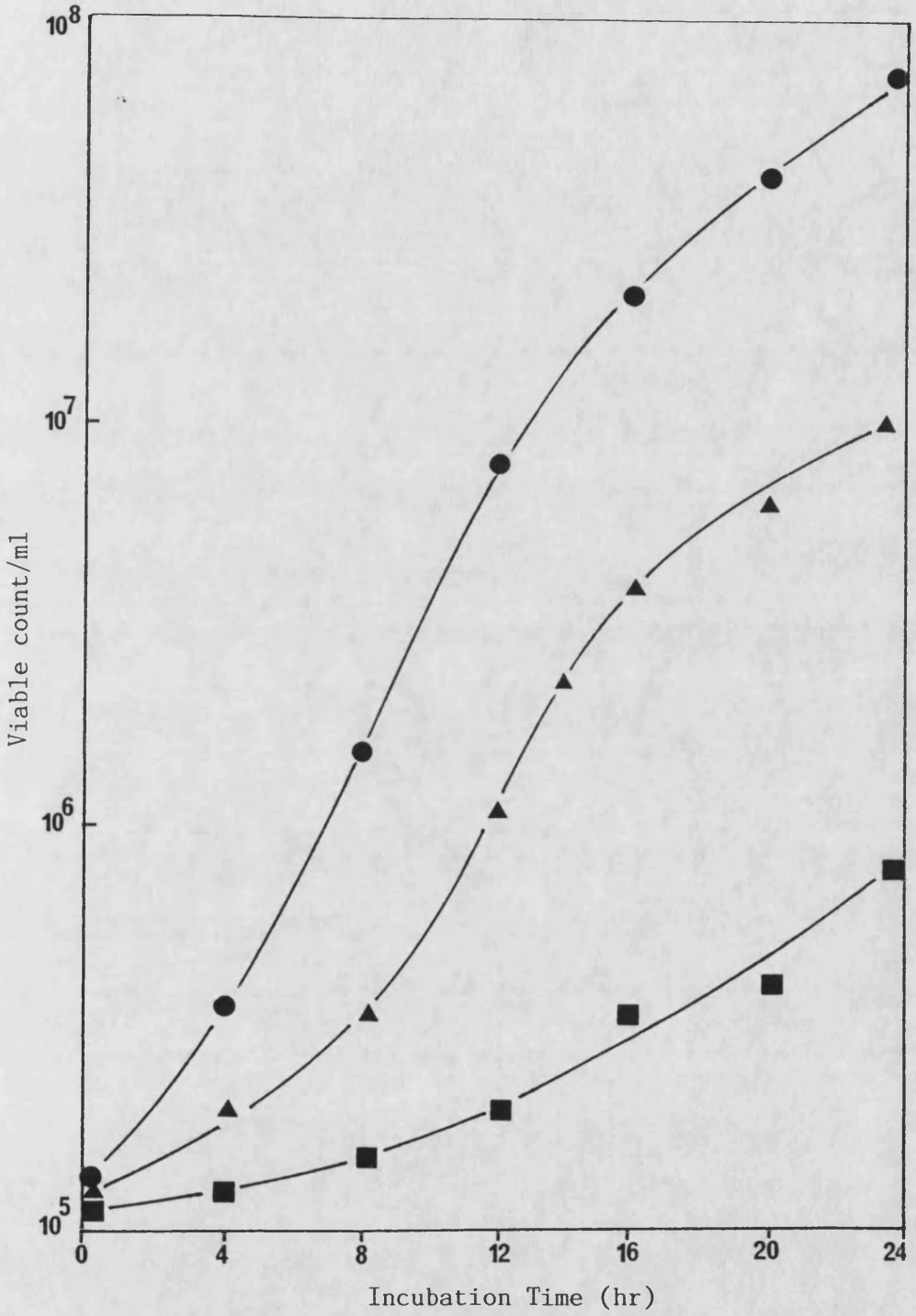


Figure 3·7 Growth curves for Candida tropicalis ATCC 32113 grown on hexadecane (●) and tetradecane (■) each as sole carbon sources in medium B. Growth with codeine (▲) is included for comparison. Incubation temperature 30°C.

Hexadecane produced the highest rate of cell growth whereas tetradecane produced a very low rate of growth with a pronounced initial lag phase. The control growth curve using codeine showed an intermediate rate of growth with an increased initial lag phase but reaching the exponential phase at about 12 hours. No viable cells were detected with decane as the sole carbon source. *Candida tropicalis* cells grown on hexadecane, tetradecane and codeine were therefore used in the subsequent study as inocula for codeine transformation studies.

3.4.5 Effect of primary culture carbon source on codeine transformation

The effect of the alkanes on the growth characteristics of *Candida tropicalis* cells was established in the previous section. Experiments were carried out to determine the effect on codeine transformation of growing the primary culture (ie. the secondary culture inoculum) on hexadecane and tetradecane as the sole carbon source.

18 hour primary cultures grown on each of the alkanes, hexadecane and tetradecane, in medium B were prepared and harvested as described in Section 3.4.4. An 18 hour primary culture grown on codeine (1.6mM) was also prepared.

Flask No.	Sole Carbon Source	Primary Culture Carbon Source
1	Codeine (1.6mM)	Codeine
2		
3	Codeine (1.6mM)	Hexadecane
4		
5	Codeine (1.6mM)	Tetradecane
6		

Table 3.3 Secondary culture flasks set up to study the effect of primary culture carbon source on codeine transformation.

10ml volumes of each of the primary cultures were used to inoculate duplicate prewarmed flasks containing codeine as sole carbon source in medium B. The secondary cultures were incubated for 24 hours at 30°C with shaking (150rpm).

Samples were taken from each of the flasks every 4 hours during the 24 hour incubation period. The samples were analysed for viable

count as described in Section 2.3.2.The norcodeine concentration of each sample was determined by subjecting it to solvent extraction and GLC analysis as described in Section 3.4.1. The viable counts determined for each culture were used to plot the growth curves shown in Figure 3.8. The norcodeine concentrations for each of the samples taken during the incubation period are shown in Table 3.4.

Sample No.	Incubation Time	Norcodeine concentration(μM)		
		Hexadecane	Tetradecane	Codeine
1	0	0	0	0
2	4	0	0	0
3	8	2.3	0	2.4
4	12	9.6	0	4.7
5	16	26.7	0	6.8
6	20	37.3	2.8	-
7	24	34.8	3.2	9.3

Table 3.4 Norcodeine concentration in secondary cultures grown in medium B containing 1.6mM codeine using inocula of *Candida tropicalis* ATCC 32113 grown on hexadecane, tetradecane and codeine carbon sources.

The results in Figure 3.8 showed that cultures using

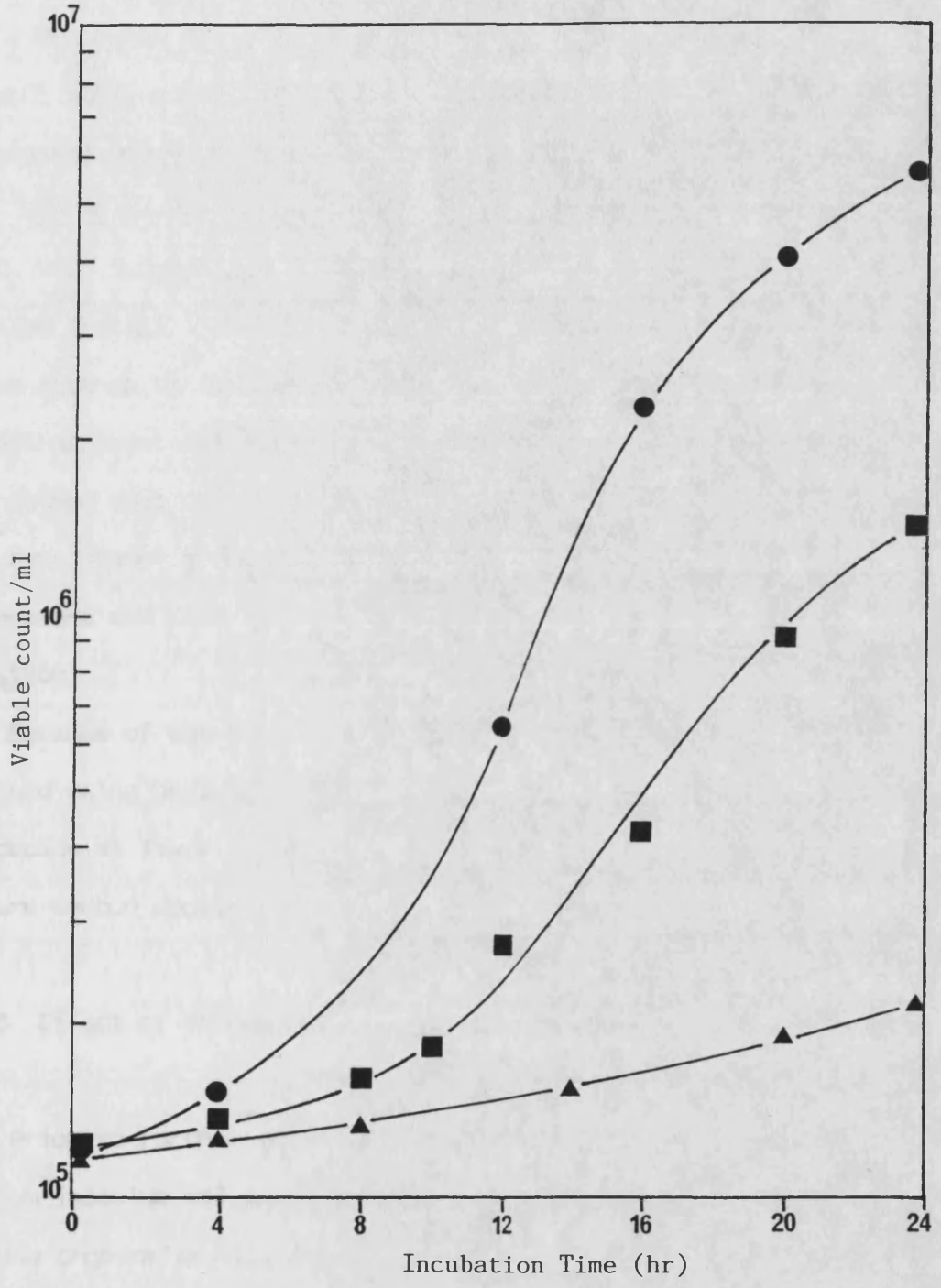


Figure 3·8 Growth curves for Candida tropicalis ATCC 32113 grown on codeine (1·6mM) as sole carbon source with inocula grown on each of hexadecane (●), tetradecane (▲) and codeine (■). Incubation temperature 30°C.

hexadecane-grown inocula were able to grow readily on codeine as the sole carbon source. The growth profile showed an initial short lag time and reached the exponential phase at 12 hours. The beginning of the stationary phase was apparent at 24 hours. Cultures using tetradecane-grown inocula showed relatively poor growth characterised by a long initial lag period and no exponential growth phase during the 24 hour incubation. Cultures using codeine-grown inocula showed the same initial lag time of 12 hours but different exponential growth rate up to 16 hours The stationary phase of growth, however, was not apparent after 24 hours incubation. The results in Table 3.4 also showed that the highest norcodeine concentrations were produced with hexadecane- grown inocula. Cultures using inocula grown on tetradecane and codeine grown inocula showed low levels of norcodeine formation.

Because of the high norcodeine concentrations and rates of growth obtained using hexadecane grown inocula, it was concluded that hexadecane at 1%w/v concentration was the most suitable primary culture carbon source for use in subsequent experiments.

3.4.6 Effect of Volume of Primary Culture Used as Inoculum

Prior to further experiments, it was necessary to establish the optimum inoculum volume to acheive maximum codeine transformation by *Candida tropicalis* ATCC 32113.

Primary 18 hour cultures were grown as described in Section 3.4.4 using 1% hexadecane as the sole carbon source. Duplicate secondary culture flasks were prepared containing codeine phosphate

(1.6mM) in chemically defined medium B (100ml). Each flask was inoculated with either 5, 10, 15 or 20ml volumes of primary culture. Inoculated flasks were incubated at 30°C with shaking (150rpm) for 36 hours. Control flasks containing only codeine (1.6mM) in medium B (100ml) were incubated under identical conditions.

At the end of the incubation period, the contents of the flasks were analysed for viable count, norcodeine concentration and pH. The data for final viable count/ml, norcodeine concentration and pH, each plotted against inoculum volume are shown in Figure 3.9.

Increasing inoculum volume from 5ml to 10ml significantly affected norcodeine production and final viable count/ml but not pH. However, there was no significant change in norcodeine production on increasing the inoculum volume from 10ml to 20ml. It was therefore concluded that the inoculum volume of 10ml used in the previous experiments was suitable for use in subsequent flask experiments with *Candida tropicalis* ATCC 32113.

3.4.7. Effect of Codeine Concentration on Codeine Transformation.

Optimisation of transformation substrate concentration is considered an important factor in microbial transformation studies (Smith and Rosazza, 1982). It was therefore essential to find the optimal codeine concentration for transformation of codeine by *Candida tropicalis* ATC 32113. The effect of codeine concentrations ranging from 0.1mM to 50mM was investigated.

Secondary culture flasks containing chemically defined medium B (100 ml) and codeine phosphate were set up as in Table 3.5.

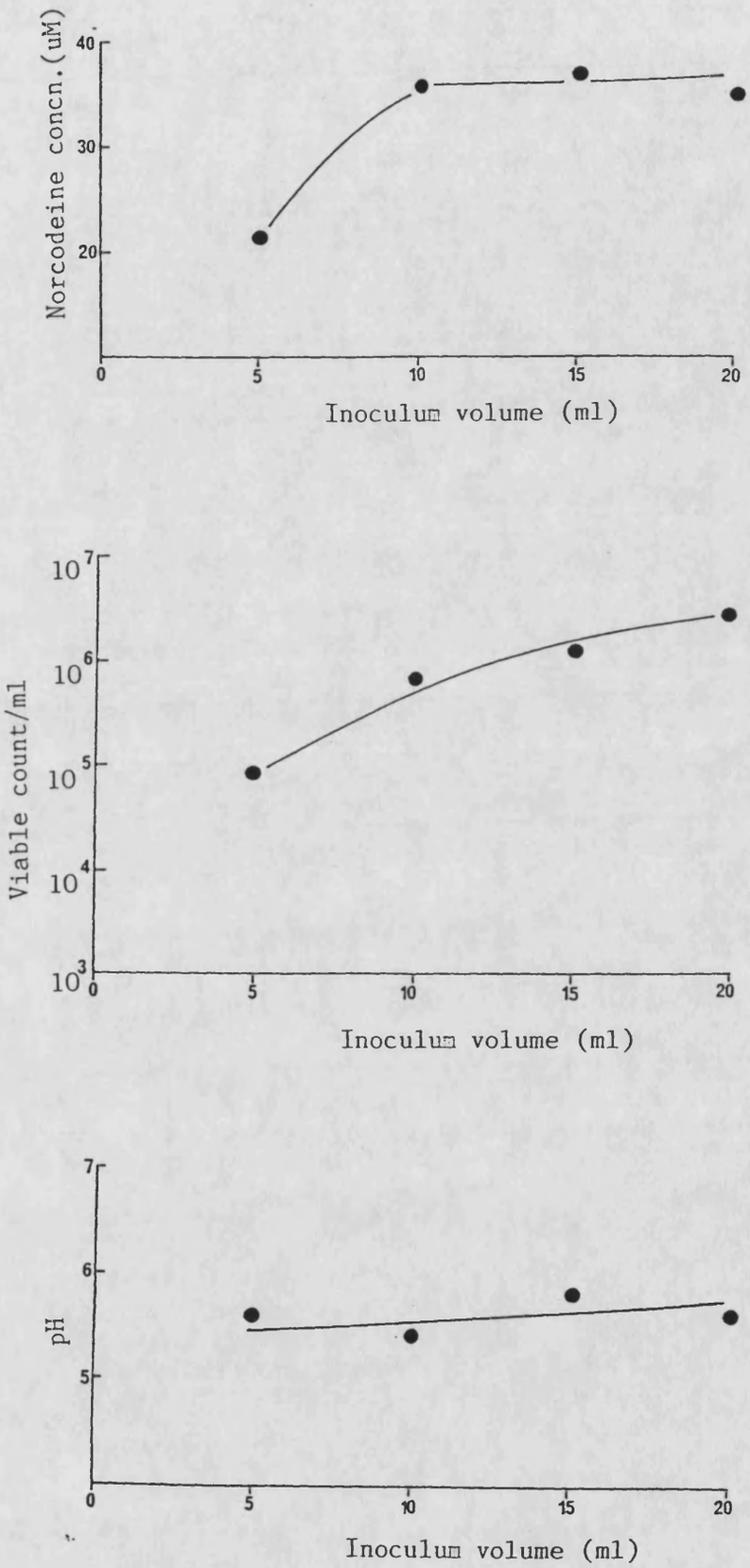


Figure 3·9 Effect of inoculum volume on norcodeine produced, final viable count/ml and pH of cultures of Candida tropicalis ATCC 32113 grown on codeine (1·6mM) as sole carbon source in medium B.

Flask No.	Codeine Phosphate added (mg)	Codeine Concentration (mM)
1	3	0.1
2	15	0.5
3	30	1.0
4	60	2.0
5	300	10.0
6	750	25.0
7	1500	50.0

Table 3.5 concentrations of codeine in secondary cultures of *Candida tropicalis* ATCC 32113 in medium B.

After prewarming at 30°C with shaking all flasks were inoculated with 10 ml of primary culture grown on 1% hexadecane. The flasks were then incubated at 30°C with shaking (150rpm) for 24 hours. At the end of the incubation period samples were taken from each flask and the final viable count determined as in Section 2.3.2. The contents of each flask were also subjected to solvent extraction as in Section 3.4.1. The extracts were analysed by GLC for norcodeine concentration as described in 3.3.3. Because of the range of codeine concentrations used and the norcodeine concentrations expected, it was essential to maintain the sensitivity of the GLC detector. Therefore the volumes of THF used for reconstitution of extracts and for GLC injection were varied as shown in Table 3.6.

Extract from Flask No.	THF Volume(ml) for Reconstitution	THF Volume (µl) for Injection
1	0.5	2.0
2	1	2.0
3	2	1.0
4	2	1.0
5	4	0.5
6	4	0.5
7	5	1.0

Table 3.6 Volumes of THF used for reconstitution of extracts and GLC injections to maintain detector sensitivity.

The final viable count/ml and final norcodeine concentration are plotted against codeine concentration in Figures 3.10 and 3.11 respectively. The data showed that maximum transformation of codeine and peak viable count/ml occured at codeine concentrations between 1mM and 2mM. This confirmed that the concentration of 1.6mM codeine selected for the preliminary studies was suitable for use in all subsequent studies.

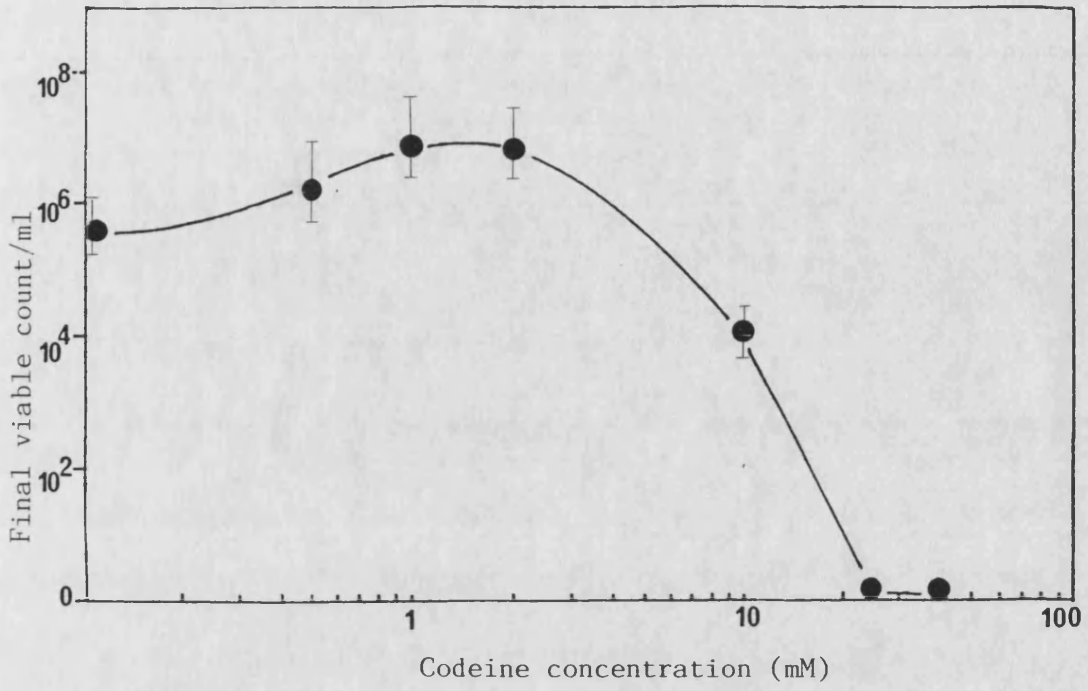


Figure 3·10 Final viable count/ml for Candida tropicalis ATCC 32113 grown on different codeine concentrations in medium B, for 24 hours. **n = 3**

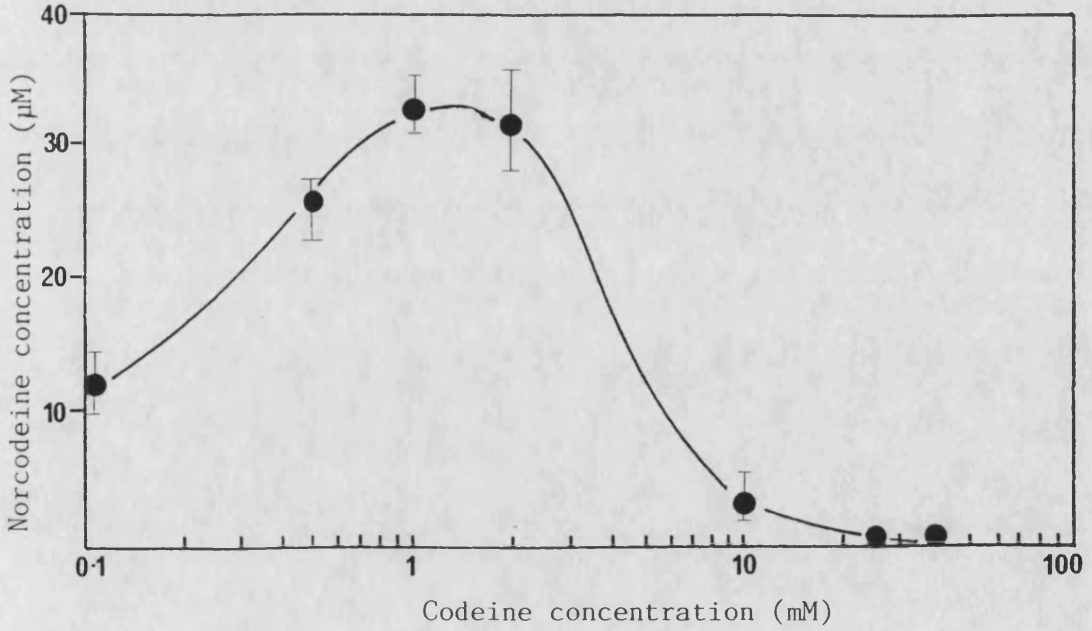


Figure 3·11 Norcodeine production by Candida tropicalis ATCC 32113 grown on different codeine concentrations in medium B for 24 hours at 30°C. **n = 3**

3.4.8. Effect of Casein Hydrolysate and Vitamins on Codeine Transformation.

Previous transformation studies with *Cunninghamella* sp have shown that casein hydrolysate as the nitrogen source increases codeine transformation (Sewell, 1982). The effect on codeine transformation by *Candida tropicalis* ATCC 32113 of casein hydrolysate as the nitrogen source in the chemically defined medium B was therefore examined.

The vitamins thiamine, pyridoxine and biotin are essential requirements for fungal growth and are included in chemically defined medium B. In order to further optimise the transformation of codeine by *Candida tropicalis* ATCC 32113 the effect of increasing the concentration of each of these vitamins was examined, as was the effect of adding folic acid.

Secondary culture flasks containing medium B were set up as described in Section 3.4.3. Where casein hydrolysate was being examined, the ammonium sulphate was omitted and replaced by 1% casein hydrolysate. The effect of the vitamins thiamine, pyridoxine and biotin was investigated by doubling their concentrations in the chemically defined medium B. The effect of folic acid was investigated by adding a filter sterilised solution in glass distilled water to medium B to a final concentration of 24mg/litre. All flasks contained codeine phosphate at 1.6 mM concentration. The flasks were inoculated with hexadecane grown inocula of *Candida tropicalis* ATCC 32113 and incubated with shaking (150 rpm) at 30°C for 24 hours. Control secondary cultures were set up containing only

medium B and codeine (1.6mM), and after inoculation these were incubated under the same conditions.

At the end of the incubation period all the flasks were analysed for norcodeine concentration by GLC as described in Section 3.3.3. Samples from each flask were also taken and the final viable count/ml determined as described in Section 2.3.2. The results obtained are shown in Table 3.7.

	Growth Final Viable Count/ml	Norcodeine Concentration (μM)
Control	5.6×10^6	34.7
Casein hydrolysate	--	-
Doubled Vitamins Concentration	4.1×10^6	12.7
Additional Folic Acid	4.6×10^6	28.3

Table 3.7 Effect of casein hydrolysate, additional vitamins and folic acid on viable count/ml and codeine transformation by *Candida tropicalis* ATCC 32113 grown in medium B containing 1.6mM codeine for 24 hours at 30°C.

Casein hydrolysate as the nitrogen source produced no detectable growth of *Candida tropicalis* cells. Doubling the concentration of thiamine, pyridoxine and biotin produced less growth and reduced transformation of codeine to norcodeine. The addition of folic acid to the medium B also reduced growth and transformation of codeine. As a consequence it was concluded that medium B should be used without any modification.

3.5. Discussion

The growth profiles shown in Figures 3.8-3.11 clearly showed that the composition of chemically defined media affecting the growth of *Candida tropicalis* ATCC 32113 when glucose or codeine were used as sole carbon sources. Maximum growth was obtained using the modified medium of Hug *et al*, 1974. Chemically defined medium A, based on the medium used for *Cunninghamella* sp by Sewell, 1982, produced poor growth using both carbon sources.

Although all components of the medium B were contained in chemically defined medium A, the poor growth in medium A may be attributed to the presence of extra salts and additional trace elements. These together with the reduction in pH during incubation, may have inhibited the reproduction of the *Candida tropicalis* cells.

The use of alkanes for growth of primary cultures showed dramatic changes. Hexadecane produced a large increase in the number of viable cells compared with glucose. Subsequent growth on codeine using hexadecane grown cells also produced increased growth and codeine transformation to norcodeine.

Cunninghamella bainieri grown on tetradecane has shown similar results of increased codeine transformation. However this was accompanied by a decrease in cell mass (Gibson, 1984). Some strains of *Candida tropicalis* have been shown to grow on tetradecane by Duppel *et al*, 1973, however the strain used in the present study showed poor growth on tetradecane and decane. This suggests that *Candida tropicalis* ATCC 32113 shows a high degree of n-alkane

specificity in chemically defined medium B. Studies by Gmunder *et al*, 1981a with *Candida tropicalis* grown on glucose, hexadecane and alkane in chemostat studies have shown that hexadecane induces production of cytochrome P-450 monooxgenase in the microsomal electron transport chain. The catalytic function of cytochrome P-450 in the N-demethylation of drugs including codeine and lanosterol by yeasts has been widely implicated (Aoyama *et al*, 1981b; Sanglard *et al*, 1986). The alkanes on being metabolized by *Candida tropicalis* are converted to fatty acids which are either directly or indirectly inserted into the lipids of the cell wall (Gmunder *et al*, 1981). Glucose grown *Candida tropicalis* however have been shown to lack a cytochrome P-450 monooxygenase capable of N-demethylation (Duppel, 1973). This may explain the reduced transformation rate of codeine observed on glucose grown cells. This catabolic repression of the codeine "N-demethylase" enzyme by glucose has also been noted by in *Cunninghamella echinulata* Sewell, 1982. Experiments to determine the effect of increasing the volume of alkane grown inoculum showed no further increase in codeine transformation.

Significant differences were, however, observed in codeine transformation when the substrate concentration was varied between 0.1 and 50 mM. There was a significant decrease in growth when the substrate concentration was increased above 2 mM. There was also a decrease in norcodeine production above 2 mM concentrations of codeine. Optimum codeine concentration was considered to be 1.5-1.6 mM, where higher concentrations were probably causing substrate inhibition of the cytochrome P450 monooxygenase leading to decreased growth and transformation. The optimum codeine concentration

compares favourably with that reproted in other studies Kunz *et al*, 1985 have found optimal codeine concentration of between 1.25 and 2.5 mM with *Streptomyces griseus* and Sewell, 1982 who found the optimal codeine concentration of with *Cunninghamella echinulata* to be 1mM. These figures are higher than that determined by Gibson, 1984 who found an optimal codeine concentration of between 0.08 and 0.2 mM with *Cunninghamella bainieri*.

Cultures of *Candida tropicalis* grown using casein hydrolysate as the sole nitrogen source replacing ammonium sulphate and yeast extract showed no detectable growth and codeine transformation. This is in contrast to findings by Sewell, 1982 where growth and codeine transformation with *Cunninghamella echinulata*were greater on casein hydrolysate than an ammonium sulphate with The addition of extra vitamins and the presence of folic acid decreased growth and norcodeine formation slightly. It was concluded therefore that the vitamins did not play significant roles in the catalysis of codeine transformation. Casein hydrolysate had a total inhibitory effect on growth and codeine transformation. This suggests that *Candida tropicalis* ATCC 32113 was unable to assimilate nitrogen in this form.

CHAPTER FOUR

CYTOCHROME P-450 YIELD AND TRANSFORMATION OPTIMIZATION FOR *Candida tropicalis* ATCC 32113 GROWN IN A FERMENTER

CHAPTER 4. CYTOCHROME P-450 YIELD AND TRANSFORMATION OPTIMISATION FOR *Candida tropicalis* ATCC 32113 GROWN IN A FERMENTER

4.1 Introduction

Cytochrome P-450 has been suggested as the enzyme responsible for the N-demethylation of codeine (Gibson, 1984). The oxidation of n-alkanes such as hexadecane by yeasts is also known to be catalysed by cytochrome P-450 (Gallo *et al*, 1973). The induction of cytochrome P-450 has been observed when hexadecane is used as the sole carbon source (Mauersberger *et al*, 1980). An important factor affecting the growth and production of cytochrome P-450 in yeasts grown on hexadecane, is the quality of dispersion of the oil phase in the aqueous phase (Gmunder *et al*, 1981b). The finer the dispersion the better the cells grow, resulting in an extended exponential growth phase in batch culture (Erickson and Nakahara, 1975). The production of cytochrome P-450 in yeasts is also dependant on the oxygen transfer rate, where low oxygen levels are known to induce the production of P-450 in alkane grown yeasts (Losinov *et al*, 1974).

In order to study the effect of mass transfer rates (of oxygen as well as substrate) on cytochrome P-450 production and cell growth, a 7 litre fermenter was used in this study. The use of the fermenter allows controlled conditions to be imposed on yeast cell growth and the effect of variation of pH, stirring intensity, temperature and oxygen availability to be studied.

It was the aim of this study to optimise the production of cytochrome P-450 produced by *Candida tropicalis* ATCC 32113. It was also the aim to optimise the codeine N-demethylation activity of the cytochrome P-450 by using codeine as the sole carbon source for growth. The effect of varying inoculum size was also investigated.

4.2 Materials

4.2.1 Chemicals and Reagents

2 M solutions of phosphoric acid and sodium hydroxide were prepared from SLR grade reagents (Fisons Scientific Apparatus, Loughborough).

Sodium dithionite (Fisons Scientific Apparatus, Loughborough).

4.2.2 Equipment and Instrumentation

An LH Series 2000 fermenter was used (L. H. Engineering Co. Ltd., Stoke Poges). The vessel (9 litre capacity, 7 litre working volume) was constructed of borosilicate glass with stainless steel top and bottom plates held in position with tie rods. The dual six blade impeller was driven directly by a 1 kW motor regulated with a thyristor variable speed controller over a range of 0-1500 rpm. Two external peristaltic pumps (for acid and alkali) were connected to the vessel *via* 3 mm PTFE tubing. Two gas flow meters (one each for nitrogen and air) were also connected. The vessel was fitted with an Ingold steam sterilisable polarographic electrode for dissolved

oxygen measurement. The temperature was measured by a steam sterilisable platinum resistance thermometer located in the bottom plate. Sparging air was passed through a steam sterilised grade AA filter (Balstron Ltd.) housed in a stainless steel filter holder and exitting gases were passed through a similar device after condensation. Control modules were supplied for temperature, stirrer speed and dissolved oxygen control. Each module was equipped with calibration and set point controls and the set point was maintained by a P10 control mechanism. Current parameter values and parameter set point values were displayed on separate digital indicators. A diagram of the fermenter vessel is shown in Figure 4.1.

4.3 Methods

4.3.1 Fermenter Methods

4.3.1.1. Preparation of the Fermenter

The fermenter vessel, all associated glassware and accessories were completely dismantled. The vessel parts were brushed clean in Link Det (Link Chemicals, London), rinsed in tap water and then rinsed in glass distilled water three times. The oxygen electrode and electrode membrane were cleaned in dilute hydrochloric acid. The electrode membrane was replaced if any damage was suspected. The pH electrode was also cleaned in dilute HCl.

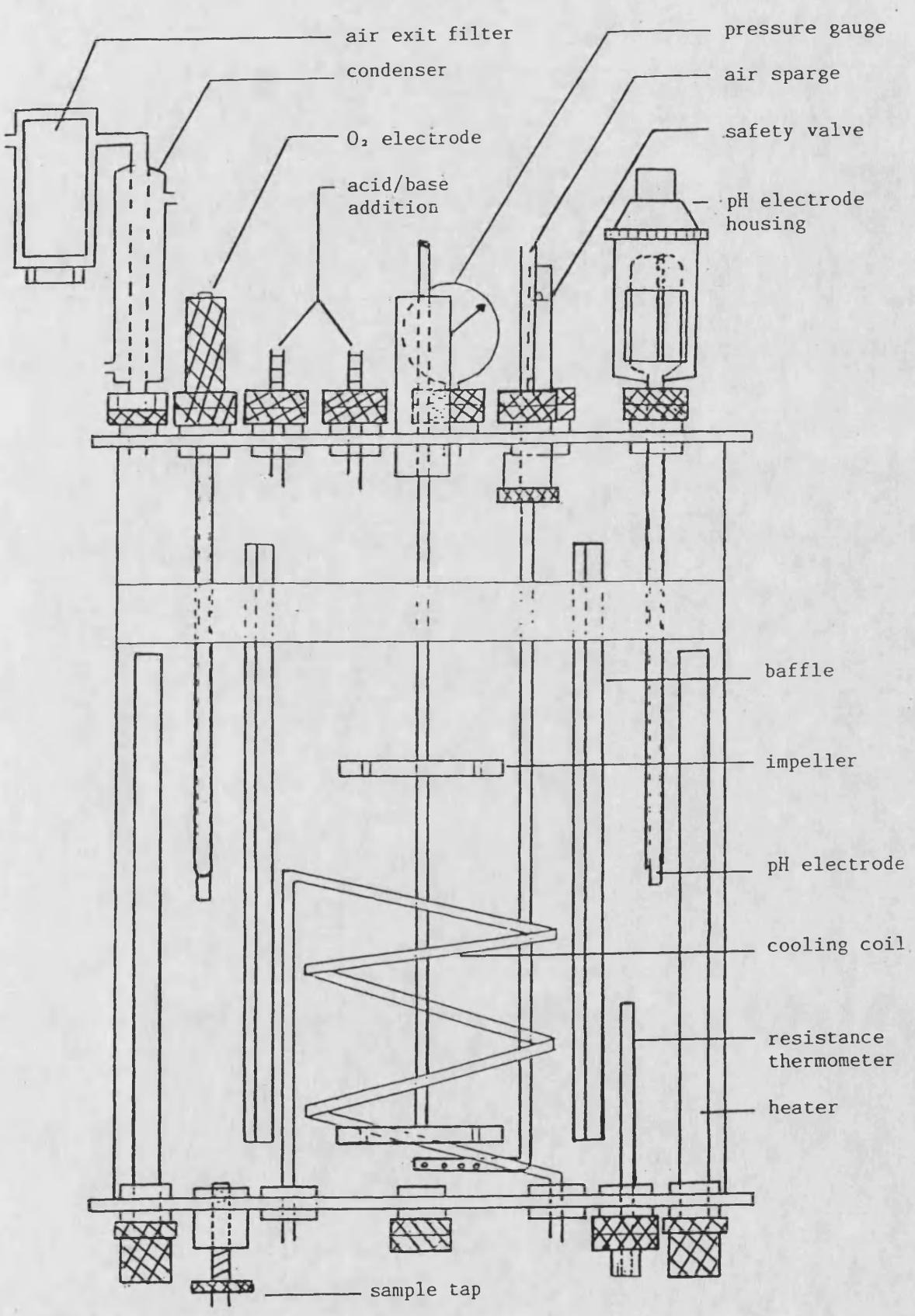


Figure 4·1 Diagram of LH 2000 series fermenter vessel

4.3.1.2. Calibration of the Automatic pH Control

The pH electrode, after washing and drying, was immersed in buffer at pH 7.0 (30°C) and the asymmetry potential adjusted to 7.00. The span and accuracy of the pH control was checked with buffers of pH 4.0 and pH 9.0.

4.3.1.3. Sequence of Fermenter Assembly

The fermenter vessel assembly was dismantled by removing the top plate and cleaned before each experiment. The vessel was reassembled by replacing the baffles into the vessel followed by replacement of the top plate. The top plate was secured onto the glass vessel by tightening the bolts on the tie rods. Before replacing the vessel onto the control module frame, the pH electrode housing, oxygen electrode and pressure gauge were fitted onto the top plate (Figure 4.1). After placing the vessel onto the control module frame, the heater probes and resistance thermometer were fixed onto the vessel at the bottom plate. The condenser, acid/base ports and air sparges were then fixed on top of the plate. All other open ports in the top and bottom plates were sealed with rubber "O" rings and threaded in stoppers prior to the sterilisation procedure.

4.3.1.4. Sterilisation

Double strength chemically defined growth medium B (3500 ml) and glass distilled water (3500 ml) were added to the fermenter vessel with pH and oxygen electrodes in place. A fresh filter cartridge was

fitted to the air exit filter unit, and all surplus ports were sealed with PTFE septa and threaded closures. A safety screen was erected around the vessel, the steam trap valve opened and the vessel contents heated to 121°C by the electrical heating probes. This temperature was maintained for 20 minutes, with the impellar blades rotating at 450 rpm, to effect sterilisation. During this procedure, the pH electrode was pressurised to 10 psi, and the water arising from the steam trap was collected and measured. The fermenter and contents were then cooled to 30°C by passage of tap water through the cooling coil. n-Hexadecane (210 ml) was filtered through a 0.45µ filter to remove particulate matter, and aseptically added to the fermenter. The measured water lost from the steam trap was replaced by an equal volume of sterile distilled water. The glass acid-base delivery tubes with reservoir closures, the inline air filter unit and PTFE tubing for delivery of acid, base and air were packed into autoclave bags and autoclaved at 121°C for 20 minutes. The glass acid and base reservoirs, each of 500 ml capacity, were closed with aluminium foil and sterilised by dry heat at 180°C for 60 minutes. The acid and base solutions were sterilised by autoclaving at 121°C for 15 minutes and then added aseptically to the respective reservoirs and the glass delivery tube and the closure fitted. The acid, base and air delivery tubes were aseptically connected to the fermenter using the threaded septum-piercing fittings provided for that purpose. The air intake tubes for the reservoirs were fitted with 0.2µ "Swinnex" filter units. 20 ml of silicone antifoam A autoclaved at 121°C for 20 minutes was aseptically added to the vessel.

4.3.1.5. Calibration of the Oxygen Electrode

Oxygen free nitrogen was sparged through the air filtration unit into the complete fermenter medium. When a constant steady dissolved oxygen readout was obtained, the reading was adjusted to zero by means of a calibration screw. The fermenter vessel containing growth medium was then sparged with air in the same manner until a constant steady reading was obtained which was then adjusted to 100% by the span adjustment screw. The entire process which was conducted at 30°C, was repeated to check the zero and 100% values until steady readings were obtained.

4.3.1.6. Inoculation

A single colony of *Candida tropicalis* ATCC 32113 from a stock agar plate was used to inoculate a sterile prewarmed solution of TSB (200 ml). After 18 hours in a shaking incubator at 30°C, 10 ml of the culture was used to inoculate 100 ml of sterile prewarmed chemically defined growth medium B containing 1% n-hexadecane. This culture was then incubated at 30°C with shaking for 18 hours. Using aseptic technique, the fermenter vessel was inoculated *via* an addition port, with 100 ml of the hexadecane-grown culture.

4.3.1.7. Sampling

Sampling was facilitated by means of a sample tap located in the base of the fermenter. This consisted of a hollow tube, of 6 mm i.d. which passed into the fermenter vessel through an O-ring seal. The

end of the tube inside the vessel was sealed except for an entrance eyelet also of 6 mm diameter at the side of the tube. In the closed position, the eyelet was retracted into the O-ring and sealed. For sampling, the tube and eyelet were projected into the interior of the fermenter vessel by means of a threaded sleeve. The first 5 ml taken was discarded and then sample volumes were collected in calibrated sterile containers.

4.3.1.8 Substrate addition

A solution of codeine phosphate (3.3 g in 50 ml) was filter sterilised (0.2μ membrane) and drawn up into a sterile 100 ml syringe. The syringe was connected *via* 10 cm of PTFE tubing to a septum-piercing addition port fitting (all previously autoclaved at 121°C for 15 minutes) and the substrate solution added to give a final codeine phosphate concentration of 1.6 mM.

4.3.2 Analytical Techniques

4.3.2.1 Assessment of growth

Volumes of 100 ml of culture were obtained aseptically at regular intervals during incubation *via* the sampling tap in the base plate of the fermenter. A volume of 2ml was taken from the 100ml sample and the viable count determined as described in Section (2.3.2). The viable count data obtained were used to construct growth curves. The remainder of the sample was then centrifuged (3500 rpm, 5 mins) to separate the cells from the supernatant. The codeine

concentration in the supernatant and the cytochrome P-450 content of the cells was determined as described below.

4.3.2.2 Determination of Codeine Transformation

The supernatant after centrifugation was subjected to solvent extraction as described in Section 3.3.2. After extracting and drying the extract was reconstituted into THF and the sample analysed for codeine and norcodeine content by GLC as described in Section 3.3.3.

4.3.2.3 Cytochrome P-450 Estimation

The cytochrome P-450 content of whole cells was determined by the method of Omura and Sato, 1964. The cells obtained after centrifugation of the culture sample were resuspended in 50 mM phosphate buffer pH 7.4. The suspension was adjusted to approximately 10 mg wet cell weight per ml. The adjusted suspension was added into a pair of matched quartz cuvettes and reduced by addition of a few grains of sodium dithionite. A baseline absorbance, over the wavelength range 500 to 400 nm was recorded for the reduced suspensions using a Pye Unicam SP600 double beam spectrophotometer. After flushing carbon monoxide gas through the test cuvette, the reduced carbon monoxide difference spectra was recorded again between 500 nm and 400 nm. The difference between the peak near 450 nm and the baseline recorded without carbon monoxide addition was used to calculate the cytochrome P-450 content of the cells. An extinction coefficient of 91 $mM^{-1}cm^{-1}$ was assumed since this value had been originally calculated and used

by Omura and Sato (1964).

4.4 Experimental

4.4.1 Effect of Oxygen Level in the Fermenter on Growth, Codeine Transformation and Cytochrome P-450 Production

The fermenter vessel was filled with chemically defined growth medium B to a volume of seven litres and sterilised as described in Section 4.3.1.5. Studies have shown the optimum pH for growth of *Candida tropicalis* to be 5 - 5.5 (Duppel *et al*, 1973, Gmunder *et al*, 1981a). The pH setting was therefore adjusted to maintain pH 5.5. The impeller speed was set to 560 rpm which was found to produce a turbid culture without excessive foaming or splashing into the air outlet.The control set points were set as follows:

Temperature 30°C

Stirrer speed 560 rpm

% dissolved oxygen level 0-100% saturation

Air sparge flow rates 0-10 litres min^{-1}

Experiments were conducted at % oxygen saturation levels ($\%O_2$) of 80%, 8% and 1%. The growth medium was adjusted to pH 5-5.5 with 2M phosphoric acid prior to inoculation and addition of the sole carbon source. Codeine phosphate at 1.6mM was used as the carbon source. The codeine phosphate was added as a filter sterilised solution in glass distilled water. The fermenter was allowed to equilibriate for 20 minutes to the required conditions and was then inoculated with an 18 hour

secondary stage culture (200 ml) of *Candida tropicalis* ATCC 32113 as described in Section 4.3.1.6. Secondary stage inocula were grown on hexadecane (1%w/v) as the sole carbon source.

Samples (100 ml) were taken at 2-4 hour intervals up to 24 hours. The samples were subjected to analysis for viable count, norcodeine concentration and cytochrome P-450 content of whole cells.

The norcodeine production and cell growth profiles for *Candida tropicalis* ATCC 32113 grown on codeine at different $\%O_2$ levels are shown in Figures 4.2 and 4.3. The cytochrome P-450 profiles for the cells are shown in Figure 4.4.

The growth profiles for *Candida tropicalis* ATCC 32113 at different $\%O_2$ levels showed that growth was affected by $\%O_2$ saturation. Growth was more prolific at 80% O_2 than at 10% and 1% O_2 saturation (Figure 4.2). Codeine transformation to norcodeine was highly influenced by $\%O_2$ saturation (Figure 4.3). A three fold increase in norcodeine formation was observed by decreasing the $\%O_2$ from 10% to 1%. Norcodeine was not detected at 80% O_2 levels. Assessment of cytochrome P-450 levels in cells during growth at steady state $\%O_2$ levels showed that no cytochrome P-450 was detectable at 80%O_2 levels. However a four fold increase in P-450 content of cells was obtained at 1% O_2 levels compared to the levels obtained at 8% O_2.

It was concluded from these studies that an $\%O_2$ level of 1% in the fermenter was optimal for norcodeine formation and cytochrome P-450

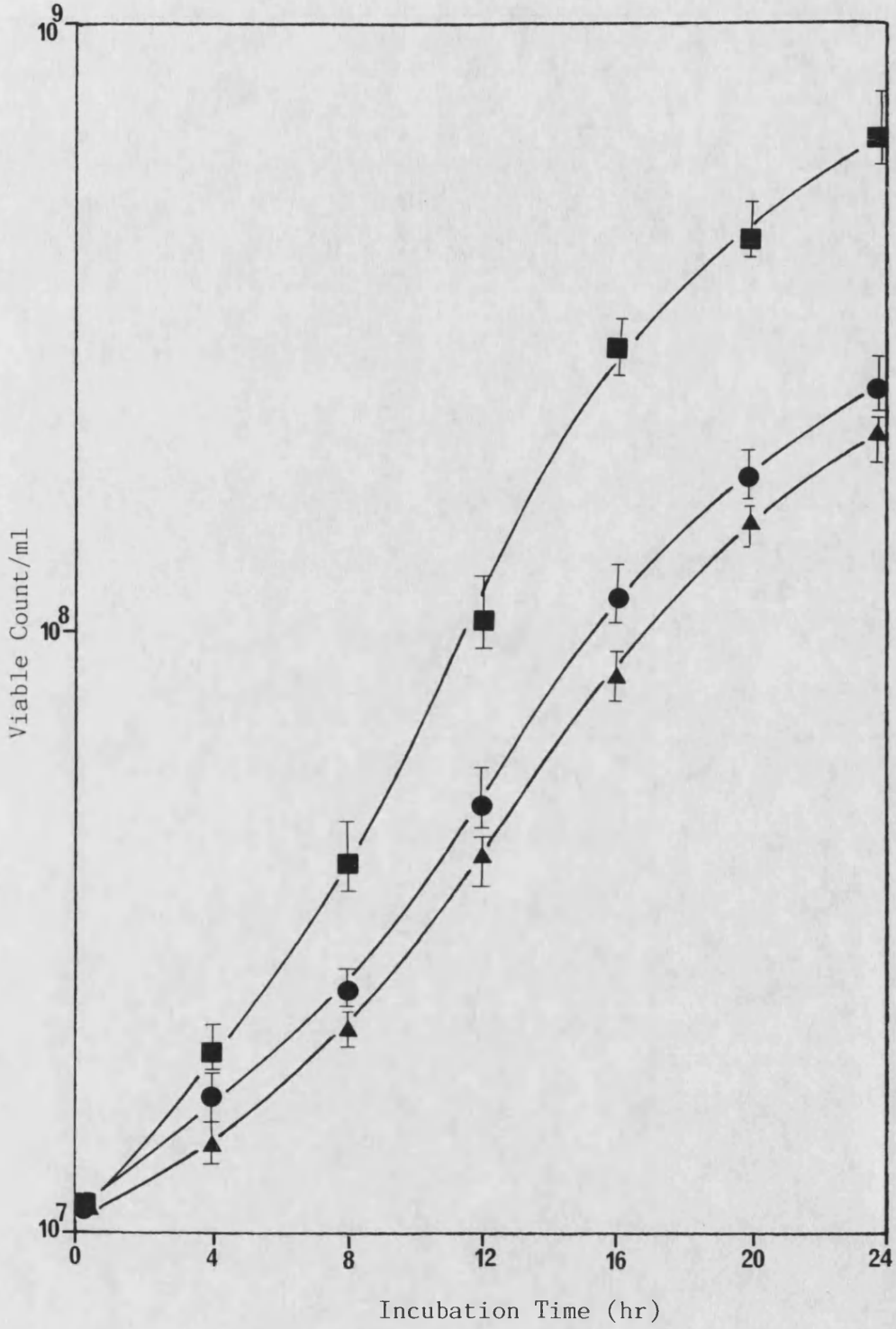


Figure 4·2 Growth curves for Candida tropicalis ATCC 32113 grown on codeine (1.6mM) as sole carbon source at $\%O_2$ saturation levels of 80% (■), 8% (●) and 1% (▲), in a fermenter at 30°C. **n = 4**

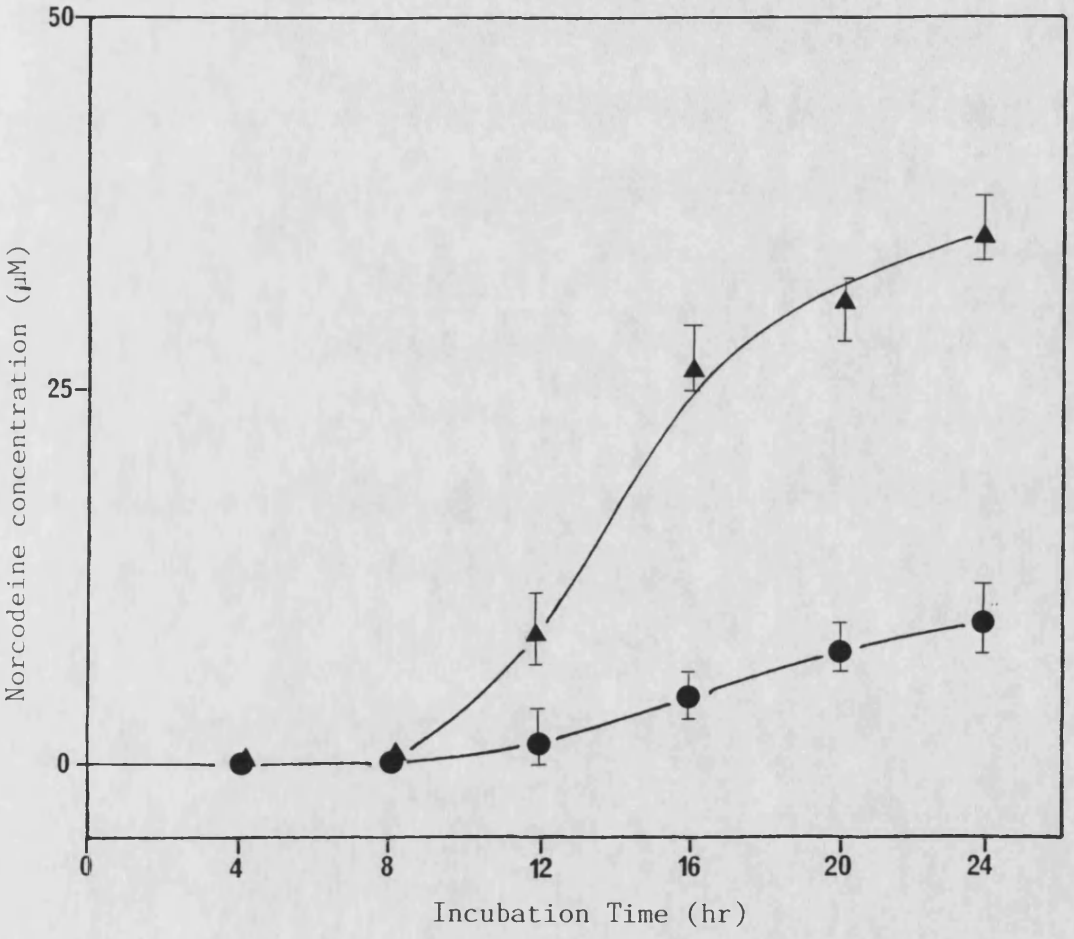


Figure 4·3 Norcodeine production profiles for Candida tropicalis ATCC 32113 grown on codeine (1·6mM) at $\%O_2$ saturation levels of 8% (●) and 1% (▲), in a fermenter, at 30°C. **n = 3**

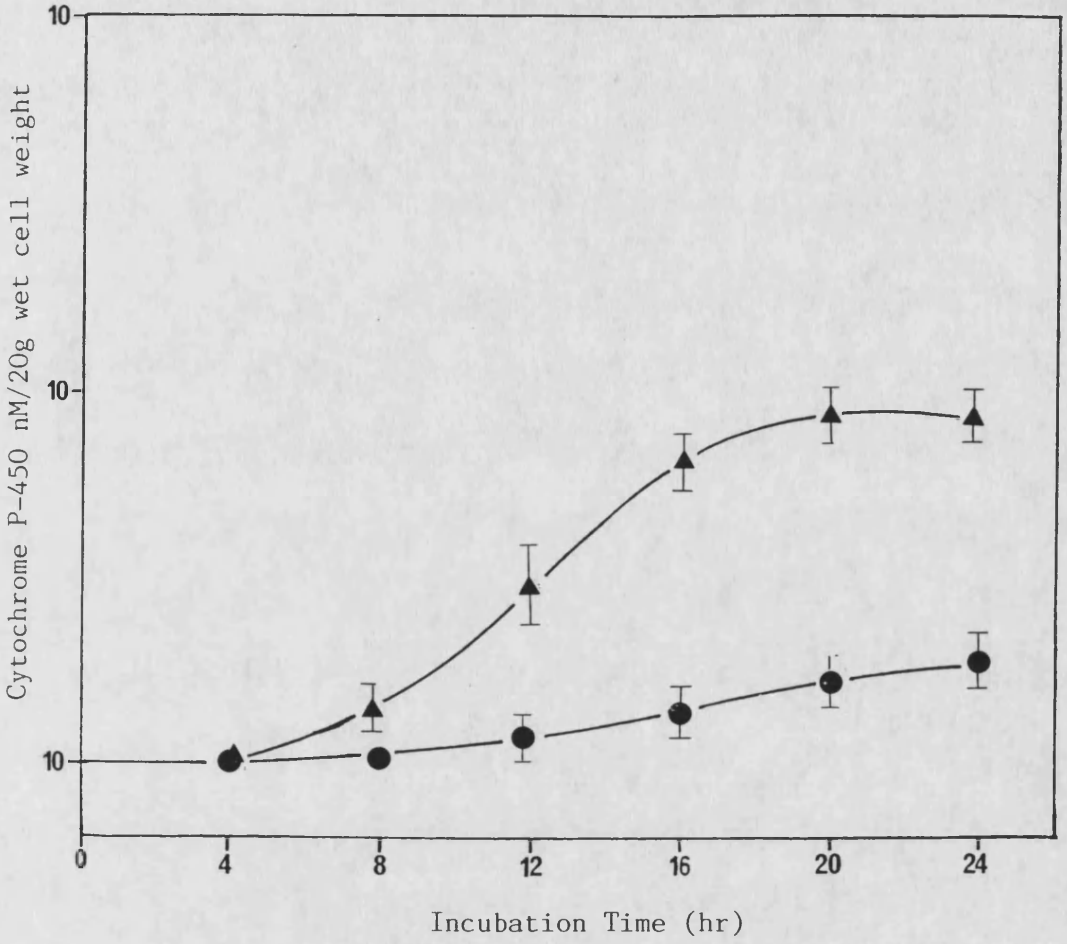


Figure 4·4 Cytochrome P-450 production profiles for Candida tropicalis ATCC 32113 grown on codeine (1·6mM) at $\%O_2$ saturation levels of 8% (●) and 1% (▲), in a fermenter, at 30°C. **n = 3**

production by *Candida tropicalis* ATCC 32113 cells grown on codeine. This $\%O_2$ level was therefore used in subsequent experiments.

4.4.2 Effect of Different Carbon Sources on Growth and Cytochrome P-450 Production in *Candida tropicalis* ATCC 32113.

The fermenter was set up containing chemically defined growth medium B and equilibriated as described in Section 4.4.1. The carbon sources investigated were hexadecane and glucose at 1%w/v concentration. The hexadecane was filtered through a 0.45µ filter to remove particles present in the oil. The glucose was sterlised by autoclaving at 121°C for 15 minutes. 18 hour secondary stage cultures of *Candida tropicalis* ATCC 32113 were grown using hexadecane (1% w/v) as the sole carbon source as described in Section 4.3.2. The secondary stage cultures (200ml) were used to inoculate the medium in the fermenter containing either hexadecane or glucose as the sole carbon sources.

Samples (100ml) were taken every 2-4 hours during the 24 hour incubation period. The cells in the samples were subjected to viable count determination and measurement of cytochrome P-450 content as described in Section 4.3.2.1 and Section 4.3.2.3. respectively. The viable counts were used to construct growth curves for cells grown on glucose and hexadecane.

The growth curves plotted against incubation time for *Candida tropicalis* ATCC 32113 grown at $1\%O_2$ saturation are shown in Figure 4.5. The growth curve for cells grown on codeine under the same conditions is included in Figure 4.5 for comparison. The typical carbon monoxide difference spectra for *Candida tropicalis* cells grown using different carbon sources under identical conditions are shown in Figure 4.6. These

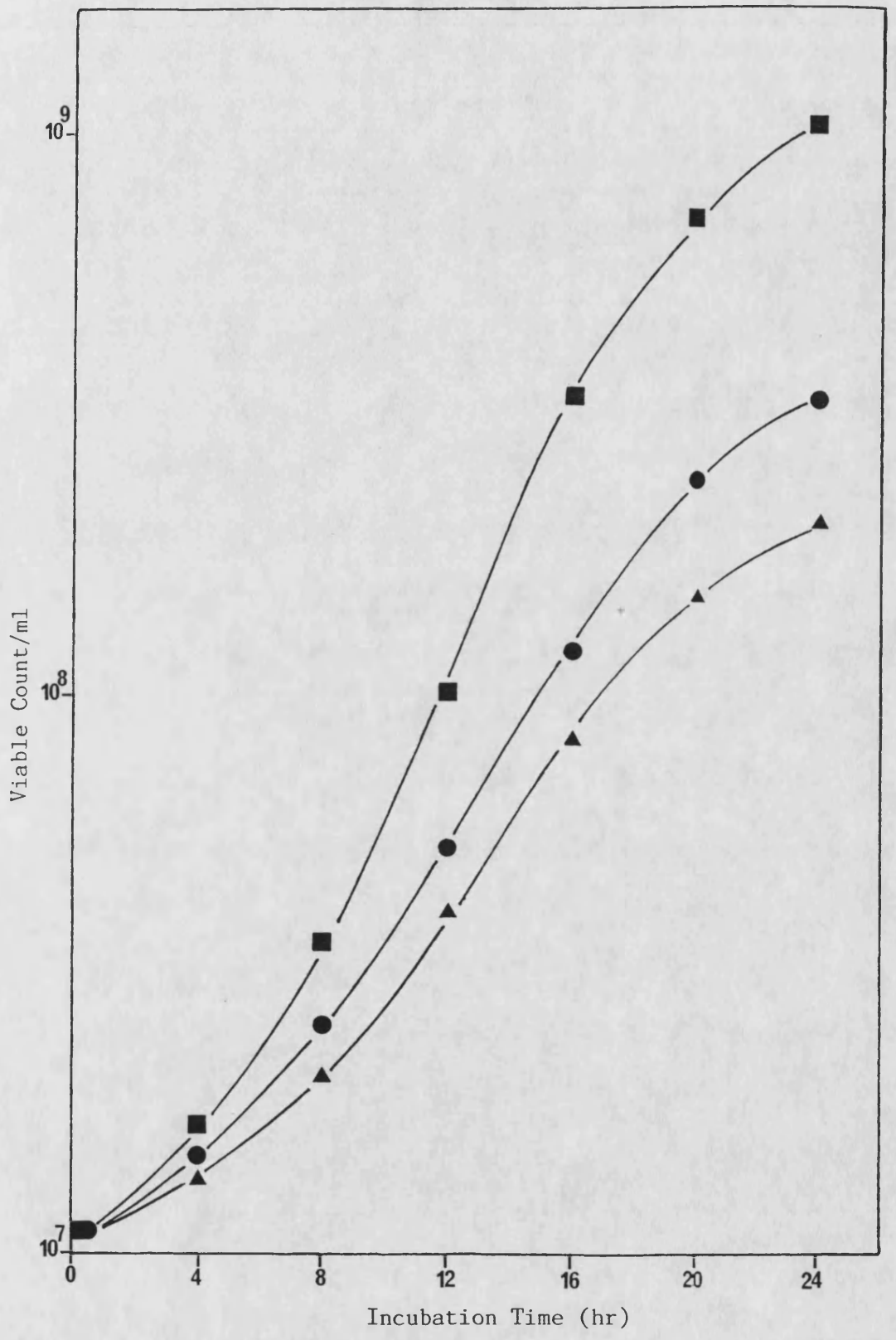


Figure 4·5 Growth curves for Candida tropicalis ATCC 32113 grown on hexadecane (1%w/v) (■), glucose (1%w/v) (●) and codeine (1·6mM) (▲) each as a sole carbon source in medium B at 1%O_2 saturation in a fermenter, at 30°C

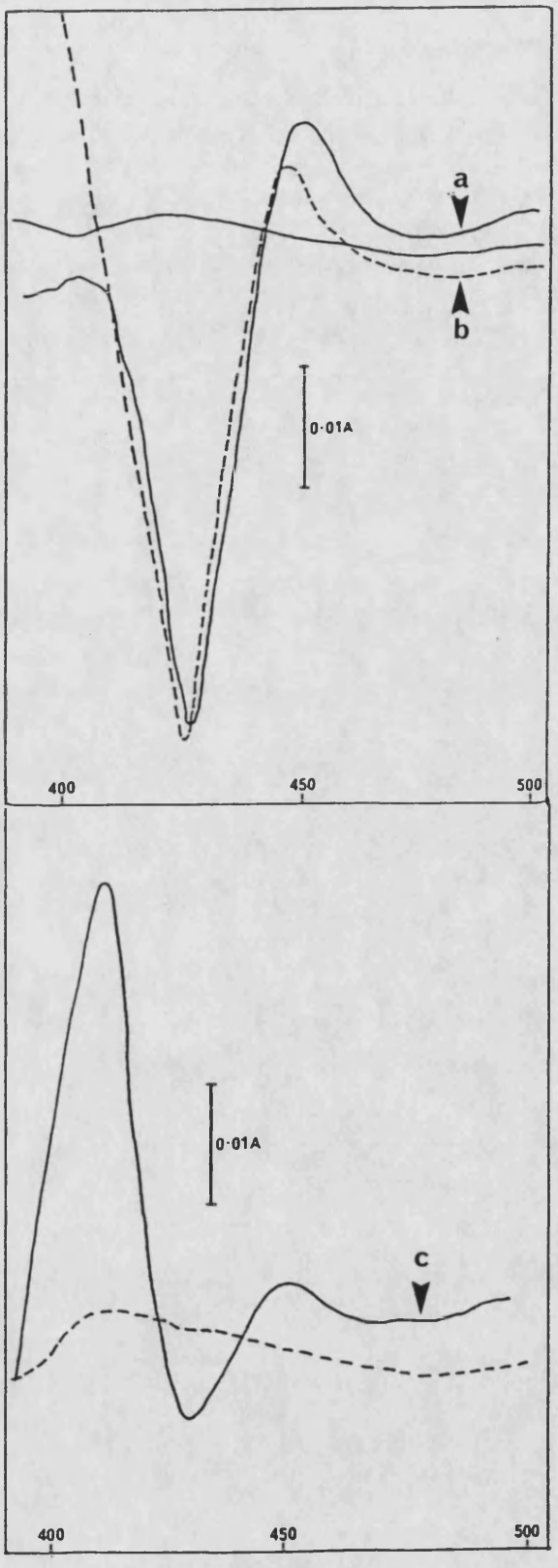


Figure 4·6 Typical carbon monoxide difference spectra for Candida tropicalis ATCC 32113 grown using hexadecane (**a**), glucose (**b**) and codeine (**c**) each as sole carbon sources in medium B at 1%O_2 saturation, in a fermenter, at 30°C.

are characterised by peaks at 450nm wavelength for cells grown on hexadecane and codeine. The glucose-grown cells did not show a peak at 450nm but exhibited a characteristic peak at 448nm. Peaks were also observed around 420nm for all carbon sources. The cytochrome P-450 production profiles for *Candida tropicalis* ATCC 32113 cells grown using the different carbon sources under identical conditions are illustrated in Figure 4.7. For glucose-grown cells, the cytochrome P-448 content of the cells was determined using an extinction coefficient of $91mM^{-1}cm^{-1}$. This value has been used by Omura and Sato (1964) for cytochrome P-448 determination of hepatic microsomal preparations. The cytochrome P-450 production profile for cells grown on codeine is included for comparision purposes.

Cytochrome P-450 production was most prolific with cells grown on hexadecane. The codeine-grown cells produced lower amounts of cytochrome P-450, while no cytochrome P-450 could be detected in cells grown on glucose. Low amounts of cytochrome P-448 were produced by glucose grown cells while cytochrome P-448 was not detected in cells grown on hexadecane or codeine. It was concluded from these studies that *Candida tropicalis* ATCC 32113 grown on hexadecane showed prolific growth and produced the largest amounts of cytochrome P-450. Hexadecane was therefore used as the sole carbon source in subsequent experiments to investigate the effect of different factors on cytochrome P-450 yield.

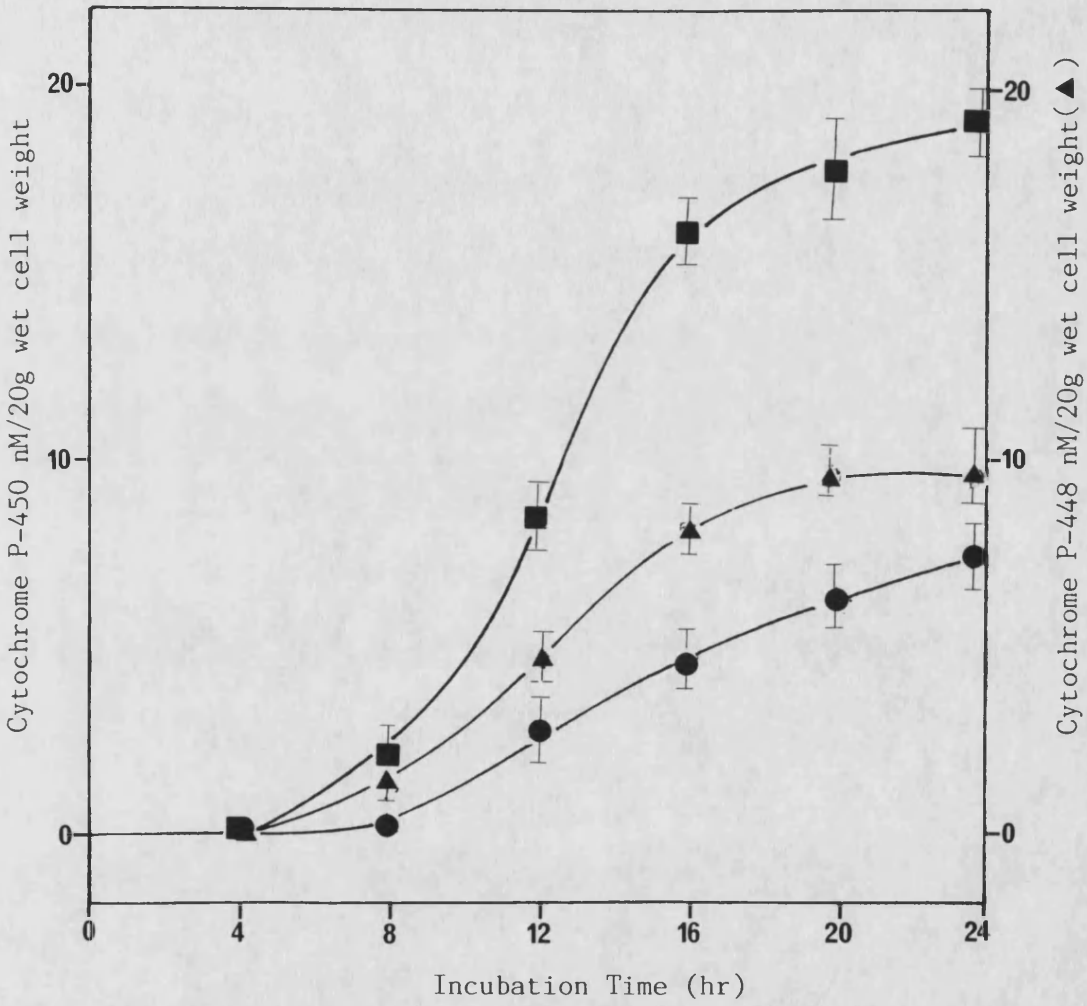


Figure 4·7 Cytochrome P-450 production profiles for Candida tropicalis ATCC 32113 grown on hexadecane (■) and codeine (▲),and cytochrome P-448 production by cells grown on glucose (●) each as sole carbon sources in medium B, at 1% O_2 saturation. **n = 3**

4.4.3 Effect of pH on Cytochrome P-450 Yield and Codeine Transformation

To further optimise cytochrome P-450 yield and codeine transformation, the effect of the pH of the medium in the fermenter on these factors was investigated.

The fermenter vessel was filled with chemically defined growth medium B to a volume of approximately seven litres and sterilised. The control set points for each experiment were:

Temperature 30°C

% dissolved oxygen saturation 1%

Stirrer speed 560-565 rpm

Air sparge flow rate <0.1 litre min^{-1}

Experiments were conducted at pH values of 4.0, 5.5, 6.5 and 7.5. The growth medium was adjusted to the required pH value by addition of either 2M sodium hydroxide or phosphoric acid. For codeine transformation experiments, a filter sterilised (0.2µ filter) solution of codeine phosphate was aseptically added to achieve the required concentration of 1.6 mM. For experiments to determine cytochrome P-450 yield, filtered volumes (0.45µ filter) of hexadecane were added to achieve a concentration of 1% w/v. The fermenter was allowed to equilibriate to the required conditions for 20 minutes and then inoculated with an 18 hour secondary culture grown on n-hexadecane (1%) (200 ml) as described in Section 4.3.1.6. The fermenter was monitored every 2-4 hours to check conditions

were maintained. Samples of 100ml were taken from the vessel every 2-4 hours for up to 24 hours.The samples (100 ml) were then analysed for cytochrome P-450 content of the whole cells and norcodeine concentration.

The effect of pH on cytochrome P-450 induction in whole cells is illustrated in Figure 4.8. The norcodeine production profiles at different steady state pH levels are shown in Figure 4.9. The experiment at a constant pH of 5.5 produced the highest concentration of cytochrome P-450 in whole cells (15nM) of *Candida tropicalis* ATCC 32113 after 24 hours incubation. A constant pH of 7.5 produced the lowest concentration at 3nM. At a pH of 5.5 the norcodeine concentration in the samples was also maximised at 38μM (Figure 4.9). However, increasing pH values up to 7.5 severely decreased norcodeine production, a maximum concentration of 6μM being detected after the same incubation period.

It was concluded from this study that a maintained pH of 5.5 was optimal for both cytochrome P-450 yield and codeine transformation. Therefore pH levels of 5.5 were maintained for all subsequent studies.

4.4.4 Effect of Stirrer Speed on Cell Growth and Cytochrome P-450 Yield

Gmunder *et al* (1981b) have shown that optimal growth of yeasts on water insoluble substrates such as hexadecane requires adequate dispersion by stirring. At an air sparge flow rate of <0.1 l min^{-1} an impeller speed of 650 rpm was found to be the maximum whereby a turbid culture was produced without excessive splashing into the air outlet of the fermenter vessel at an air sparge flow rate of <0.1 lmin^{-1}.

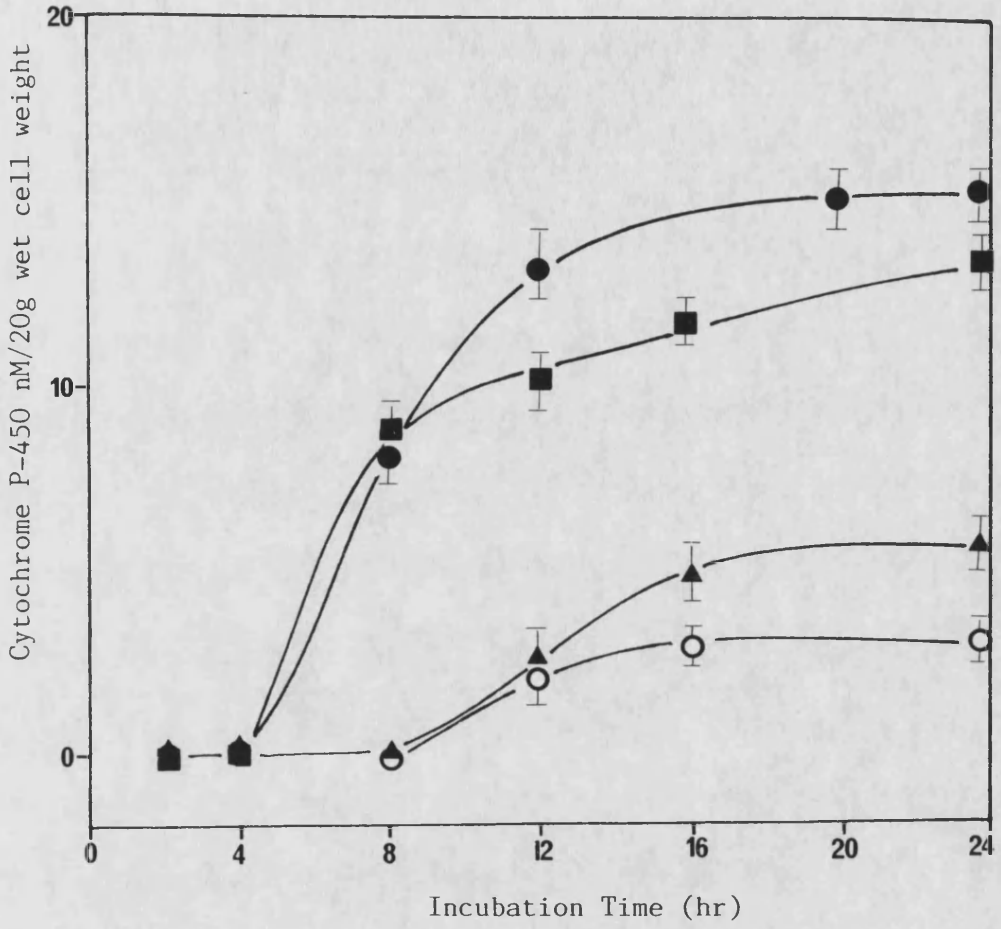


Figure 4·8 Cytochrome P-450 induction profiles for Candida tropicalis ATCC 32113 grown on hexadecane at 1%O_2 saturation at culture pH levels of 4·0 (■), 5·5 (●), 6·5 (▲) and 7·5 (○). Incubation temperature 30°C. n = 3

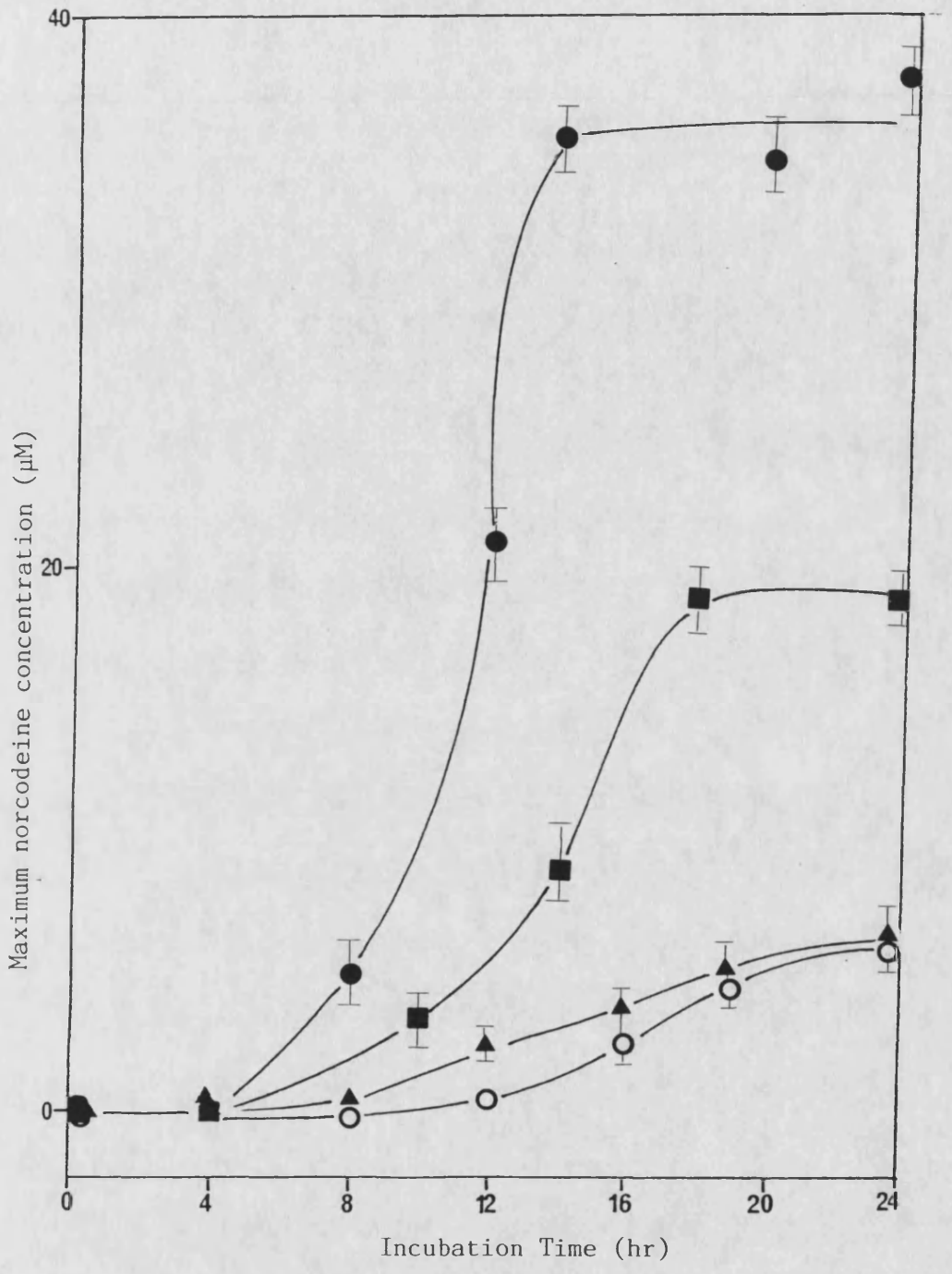


Figure 4·9 Norcodeine production profiles for Candida tropicalis ATCC 32113 grown on codeine (1·6mM) at pH levels of 4·0 (■), 5·5 (●), 6·5 (▲) and 7·5 (○). Incubation temperature 30°C. **n=3**

The fermenter vessel was filled with chemically defined medium B and the contents sterilised as described in Section 4.3.1.5. The control set points for each experiment were as in Section 4.4.3.

Experiments were conducted at stirrer speeds of 500, 550 and 650 rpm using hexadecane (1%) as the sole carbon source. The growth medium was adjusted to pH 5.5 by addition of 2M phosphoric acid prior to inoculation with an 18 hour secondary cultures of *Candida tropicalis* ATCC 32113 grown on hexadecane (1%).

The induction of cytochrome P-450 in whole cells grown at the various stirrer speeds is illustrated in Figure 4.10. The growth curves obtained in the same experiments are shown in Figure 4.11. Variation in stirrer speed between 500 and 650rpm did not significantly affect cytochrome P-450 production or growth of cells on hexadecane. It was therefore concluded that a stirrer speed of 550rpm was sufficient for use in subsequent experiments.

4.5 Discussion

The seven litre fermenter provided a very useful method for studying the growth of *Candida tropicalis* cells under controlled conditions. This control of growth conditions provided further optimisation of production of the cytochrome P-450 and codeine N-demethylation.

The data presented in Figure 4.2 showed that decreasing the steady state levels of oxygen saturation (%O_2) in the medium increased the growth

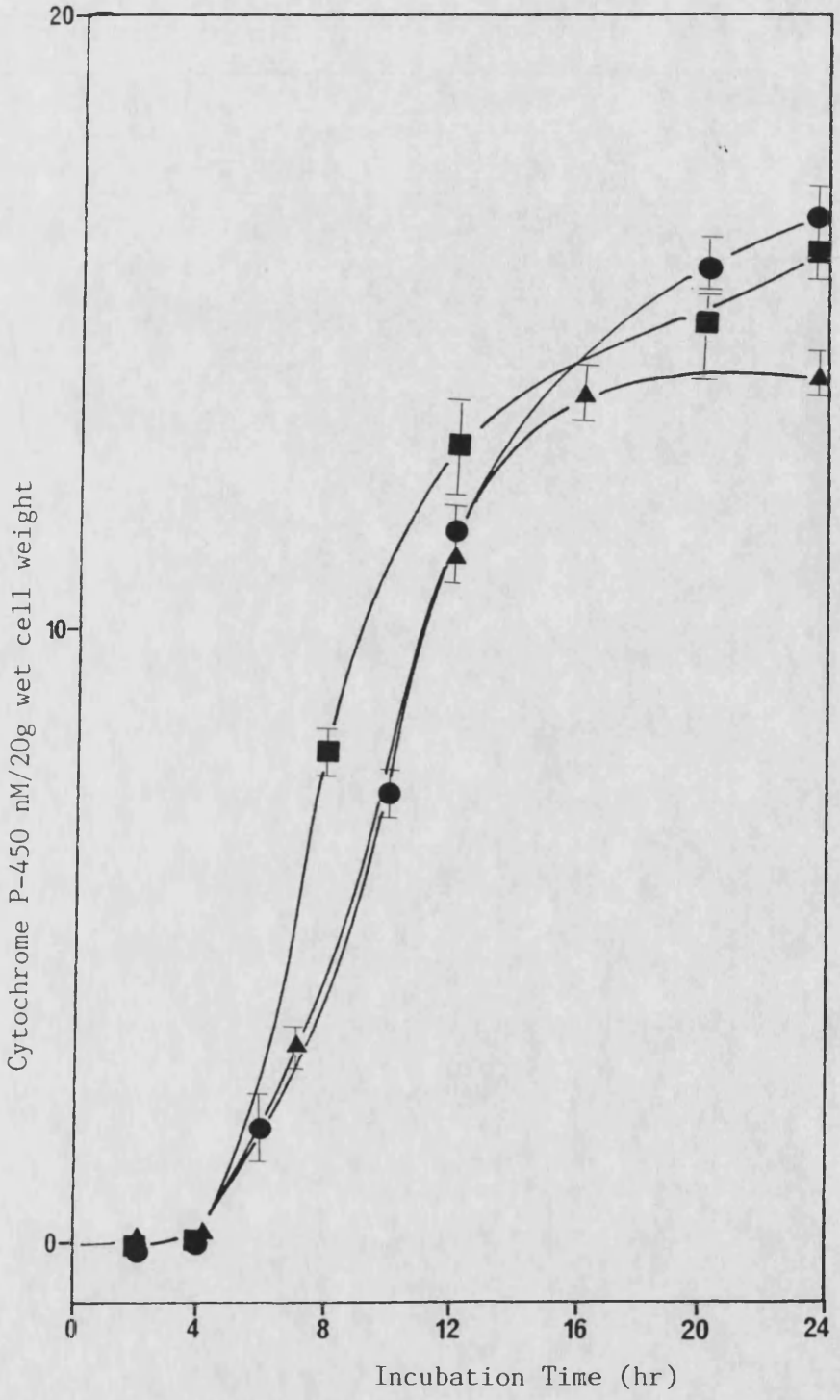


Figure 4·10 Induction of cytochrome P-450 in Candida tropicalis ATCC 32113 grown at various stirring speeds (rpm) on hexadecane(1%) at 1%O_2 saturation.
500rpm (■), 550rpm (▲), 650rpm (●).
n=3

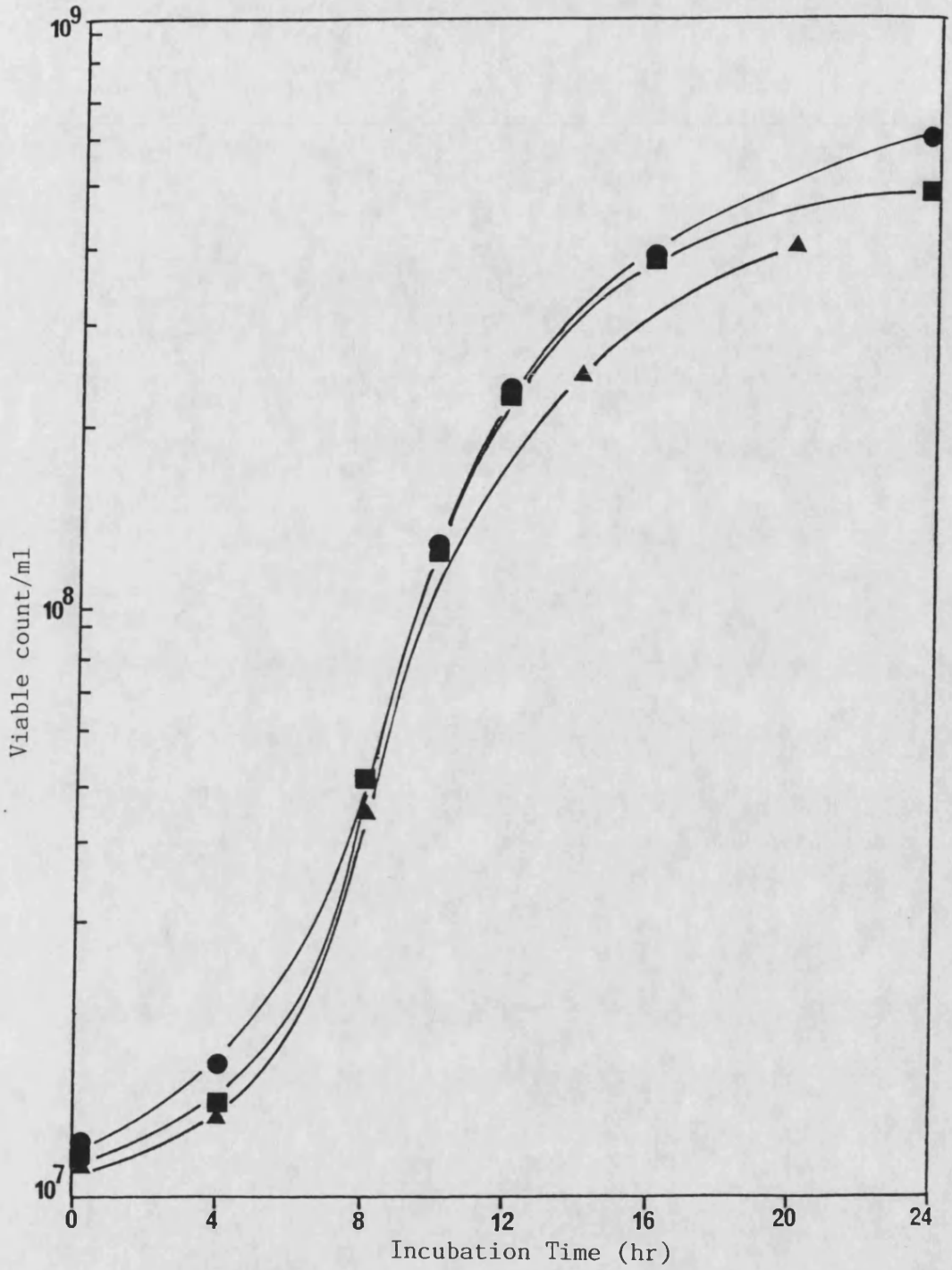


Figure 4·11 Growth curves for Candida tropicalis ATCC 32113 grown at different stirring speeds on hexadecane (1%) at 1%O_2 saturation.
500rpm (▲), 550rpm (●), 650rpm (■).

rate of cells, using codeine as the carbon source. Further investigation using steady state $\%O_2$ levels (Figure 4.3) confirmed that a low $\%O_2$ level optimises production of cytochrome P-450 whereas levels above 8% O_2 produced no detectable cytochrome P-450 in cells. The induction of cytochrome P-450 at low $\%O_2$ levels has been observed by several authors for different yeast strains capable of growing on n-alkanes (Gallo *et al*, 1976; Tittelbach *et al*, 1976; Gilewicz *et al*, 1979; Mauersberger *et al*, 1980 and Gmunder *et al*, 1981a). The transformation of codeine to norcodeine was also optimised at low $\%O_2$ levels (Figure 4.5). This supports the implication of cytochrome P-450 being involved the N-demethylation reaction. Therefore low $\%O_2$ levels in the medium may be inducing P-450 production , and consequently increasing codeine N-demethylation by *Candida tropicalis* cells.

The studies using different carbon sources showed that hexadecane was a potent inducer of cytochrome P-450 and cell growth. The adsorption of hexadecane onto the cell surface and diffusion through the cell wall towards the cell membrane is known to be much faster than the flux through the cell (Kappeli and Fiechler, 1980). The actual uptake of hexadecane has been postulated to occur by pinocytosis (Meissel *et al*, 1973) but a simple diffusion process remains a strong possibilty. The rate limiting step of hexadecane uptake and hydroxylation is located at the first oxidation step and the enzyme catalysing the reaction is cytochrome P-450 (Gmunder*et al* 1981b). Low $\%O_2$ levels may be influencing the hydroxylation rate of the cytochrome P-450 system. The cells may then be responding to the decreased hydroxylation rate by increasing the level of cytochrome P-450 in the cells. This may explain the lack of oxygen enhancing the

formation (or inhibiting degradation) of cytochrome P-450 (Figure 4.4). Yeast cells grown on the degradation products of hexadecane (hexadecanol and palmitic acid) have been shown to contain very little cytochrome P-450 (Mauersberger *et al*, 1980). Tetradecane has been shown to be a strong inducer of cytochrome P-450, but in other strains of *Candida tropicalis* (Duppel *et al*, 1973). The growth of the *Candida tropicalis* cells, however, was little affected by high $\%O_2$ levels in the medium (Figure 4.2). This is expected because at low oxygen supply rates the cells grow as fast as the supply rate allows i.e. growth of cells is limited by oxygen availability. Therefore, at higher oxygen levels there is no growth limiting factor and higher growth rates, than the ones obtained in Figure 4.2 would be expected. Studies using *Cunninghamella* sp. have shown that cytochrome P-450 is induced in the fungal cells at high $\%O_2$ levels (40%) in the medium (Gibson, 1984).

The glucose grown yeast cells showed a totally different carbon monoxide difference spectrum than that for hexadecane grown cells (Figure 4.6). The peak observed at 446-448 nm for glucose, suggests that glucose induces a totally different type of cytochrome. Mauersberger *et al*, 1980 detected no cytochromes around the 450 nm range with *Candida guilliermondii* grown on glucose. The different "type" of cytochrome P-450 present in *Candida tropicalis* cells grown on glucose has been demonstrated to show specific reaction characteristics (Sanglard *et al*, 1986). The study showed that cytochrome P-450 was not detected in whole cells but in microsomes where the reduced carbon monoxide difference spectrum shows a peak at 447 nm (Sanglard *et al*, 1984). Decreasing the $\%O_2$ in the medium had a similar effect on glucose grown cells by increasing the P-448

content of the whole cells.

Earlier preliminary experiments in this study attempting to use glucose grown inocula for hexadecane growth studies showed that although a little growth was detected the yeast cells contained no detectable cytochromes in the 450 nm range. This suggests that glucose at low $\%O_2$ levels may be inducing a different type of cytochrome in the monooxygenase system of the *Candida tropicalis* cells.

Yeast cells grown on codeine phosphate showed the presence of cytochrome P-450 (Figure 4.6). Although growth at low $\%O_2$ levels showed little induction of P-450, an increase in the production of norcodeine was observed (Figure 4.3). This suggests that codeine at low $\%O_2$ levels is readily taken up by the yeast cells and induces a more potent form of cytochrome P-450 in the cells, although in low quantities. This may explain the relatively low levels of P-450 detected in the codeine grown cells. Preliminary experiments with *Candida tropicalis* cells showed poor growth and no detectable cytochromes in the 450 nm region, when using glucose grown inocula for codeine growth studies. This again suggests that glucose is inducing a different type of cytochrome in the monooxygenase system of the yeast cells and the cells are then unable to utilise subsequently a different type of carbon source.

On investigating the effect of steady state pH levels, the cytochrome P-450 and maximum norcodeine concentration were optimised at pH 5.5. The lowest cytochrome P-450 and norcodeine concentration were detected at pH 6.5 and 7.5. The time taken to reach the exponential region of the curves of both cytochrome P-450 and norcodeine formation was also much shorter at pH 5.5. It has been shown that increasing the pH up to 7.5 increases the

percentage of unionised codeine base. Unionised codeine is thought to partition into the cell more readily (Sewell,1982). Maximal codeine transformation was occurring at pH 4.0 and 5.5 where the ionised form of codeine predominates. This suggests that the yeast cells may be taking up the codeine into the cell membrane by another process such as pinocytosis. This mechanism has already been postulated for the uptake of hexadecane by yeast cells (Meissel *et al*, 1973). This may explain the effectiveness of hexadecane grown inocula in codeine transformation experiments. The pinocytosis process may be working in conjunction with a simple diffusion process and at pH levels above 5.5 the process is inhibited. The low norcodeine formation observed at pH 4.0 may be attributed to a less potent form of cytochrome P-450 being induced at pH 4.0.

Variation of stirrer speed between 500 rpm and 650 rpm had little effect on the cytochrome P-450 content in the cells or on the growth rate of the cells. At 550 rpm a slightly faster rate of P-450 induction was observed.

The length of the exponential growth phase of yeasts on n-alkanes is related to the degree of dispersion (Erickson and Nakahara, 1975). This has also been correlated to the stirring intensity (Erickson and Nakahara, 1975). The growth rate of *Candida tropicalis* and the cytochrome P-450 production were optimised at 550 This implies that the degree of dispersion was already maximised in the culture at 550 rpm and this stirrer speed was maintained for all further experiments.

CHAPTER FIVE

N-DEMETHYLATION STUDIES USING CELL FREE EXTRACT FRACTIONS PREPARED FROM *Candida tropicalis* ATCC 32113

CHAPTER 5. N-DEMETHYLATION STUDIES USING CELL FREE EXTRACT FRACTIONS PREPARED FROM *Candida tropicalis* ATCC 32113

5.1 Introduction

The studies in the previous section showed that growth of *Candida tropicalis* ATCC 32113 grew readily in the presence of codeine, and codeine increased the N-demethylation activity of cytochrome P-450. The cytochrome P-450 production in cells grown in the presence of codeine was optimised by using oxygen saturation levels of 1% in the medium and an inoculum that had been grown with 1% hexadecane as the carbon source. After the detection of cytochrome P-450 in cell free extracts of yeast cells (Lindenmayer and Smith, 1964), Ishidate *et al* (1963) were the first to report that the cytochrome P-450 was bound to a particulate fraction, the microsomes. To isolate microsomes, cell breakage is required. Disruption of yeast cells may be achieved mechanically or by spheroplasting the cells enzymatically before a mild mechanical treatment or osmotic shock is used to release the cell contents. Besides the use of enzymes for lysing yeast cell walls (Scott and Schekman, 1980), other chemicals such as Triton X-100 have been used to ease the lysing of the cell (Spatz, 1971). Other methods of cell disruption have included pressure using a French Pressure Cell (Yoshida *et al*, 1974), a Vibromill (King *et al*, 1984) and a Dynomill (Schunck *et al*, 1978). To prepare cell free extracts from *Cunninghamella sp*, Gibson (1984) used a Braun Cell ballmill, and this technique was adopted for this study to prepare cell free extracts from *Candida tropicalis*.

Techniques have been developed to prepare microsomal fractions from cell free extracts. Differential centrifugation has been commonly used (King *et al*, 1984; Schunck *et al*, 1978 and Yoshida *et al*, 1974) A mitochondrial fraction is first formed by one or two centrifugation steps. Microsomes are then sedimented by further high speed centrifugation, and purification of cytochrome P-450 is accomplished by density gradient centrifugation (Gallo *et al*, 1971). Density gradient centrifugation, however, yields low amounts of cytochrome P-450 and contamination with other membrane fragments is known to occur. A technique was developed by Schenkman and Cinti (1972) using $CaCl_2$ aggregation to prepare microsomes from mammalian liver preparations and this technique has been adopted by Kappeli *et al*, 1982 to prepare microsomes from yeast cell free extracts. This technique of $CaCl_2$ aggregation was adapted in this study to prepare enriched cell free extracts from *Candida tropicalis* ATCC 32113. Studies with *Candida* sp have shown this cytochrome P-450 enrichment technique to be fast, suitable for scale up and to suffer from very low contamination (Laurila *et al*, 1984).

The aim of the experiments reported in this section was to evaluate the methods for cell disruption and enrichment of cytochrome P-450 in *Candida tropicalis* ATCC 32113. The activity, characteristics and stability of the enriched cytochrome P-450 was also investigated.

5.2 Materials

5.2.1 Chemicals and Reagents

Triton X100, G250 Coomassie blue dye, Sodium dithionite (Analar), Semicarbazide HCl (Analar), Carbon monoxide gas, Sodium chloride (Analar), Ferrous sulphate (Analar), Calcium chloride (Analar), Potassium chloride (Analar), Ethylene diamine tetraacetic acid disodium salt (EDTA) and polyethylene glycol (Analar) were all obtained from BDH Ltd, Poole. Ketoconazole and Miconazole were supplied by Janssen Pharmaceuticals. Bovine serum albumin (BSA), Xanthine oxidase, NADPH (tetrasodium salt), NADH (disodium salt) Sorbitol, Dithiothreitol (DTT) and Crude lyticase 800 units/mg. were purchased from the Sigma Chemical Co. Ltd. Nitrogen gas (oxygen free) was purchased from British Oxygen Ltd and Tris HCl from (Aldrich).

Sorbitol and phosphate buffers were prepared freshly before use using Analar grade reagents. G250 Coomassie blue dye was prepared as a 0.06% solution in 3% perchloric acid (Fisons Scientific Apparatus). The dye was filtered to remove undissolved material. The stock dye solution was diluted with 3% perchloric acid solution to achieve an absorbance of 1.5-2.0 at 595 nm. The stock solution was stored at room temperature and fresh solutions prepared on 14 day intervals.

5.2.2 Buffer Solutions

Sorbitol buffer solution A, 0.65M (for storing *Candida tropicalis* cells); (g) Tris HCl 1.58, Sorbitol 118.4, DTT 15.42

mg, EDTA 37.2 mg, glass distilled water to 1000 ml.

Sorbitol buffer solution B, 2M (for storing *Candida tropicalis* spheroplasts); (g) Tris HCl 1.58, Sorbitol 364.3, DTT 15.42 mg, EDTA 37.2 mg, glass distilled water to 1000ml.

5.2.3 Equipment and Instrumentation

Braun Homogeniser; Braun ballmill cell homogeniser Model MSK, operated at 2000 rpm radial frequency with capillary coolant (CO_2), Glasperlen.

Centrifuge; MSE High Speed 18 with 6x250 ml and 16x15 ml rotors, MSE Scientific Instruments Ltd.

Centrifuge tubes; Polypropylene 15 ml and 250 ml capacities with closures, MSE Scientific Instruments Ltd.

Glass beads; 0.45-0.50 mm diameter, Glasperlen.

Incubation tubes; 15 ml capacity glass with screw caps, Fisons Scientific Instruments Ltd.

Shaking bottle; 75 ml capacity Pyrex glass with ground glass stopper and retainer, Glasperlen.

Sonicator; variable frequency with 8 mm tungsten probe, Ultrasonics Ltd.

Spectrophotometer; Perkin Elmer P5505 digital readout , Perkin Elmer Ltd.

Water baths; recirculating, Grant Instruments Ltd, Cambridge.

All glassware used for cell free extract studies was washed in Link Det 710 (Link Chemicals, Berkhamstead, UK.) and tap water until

clean. The glassware was then rinsed and soaked in fresh glass distilled water for 12-14 hours, dried, and sterilsed using dry heat at 180°C for 60 minutes. All polypropylene centrifuge tubes were washed in Link Det 710 and thoroughly rinsed two times with tap water and then glass distilled water and left to soak for 12-14 hours. The tubes were dried at 45°C and sterilised by autoclaving at 121°C for 15 minutes.

5.3 Methods

5.3.1 Cell Free Extract Procedures

5.3.1.1. Growth and harvesting of cells

Candida tropicalis ATCC 32113 cells were cultivated in the seven litre fermenter according to the procedures and incubation conditions described in Chapter 4. All inocula were grown in shake flasks using hexadecane (1%) as the sole carbon source. Cells were cultivated in the fermenter in chemically defined medium B containing either codeine (1.6mM) or hexadecane (1%) as sole carbon sources. Cells were harvested after 24-36 hours incubation, by centrifugation (3500 rpm, 5 min) and washing with ice cold sorbitol buffer A at pH 7.4. Harvested cells were either used immediately or stored in polypropylene vials under liquid nitrogen. Stored cells were thawed at room temperature immediately prior to use. All extraction

procedures were conducted at 3-4°C unless otherwise stated.

5.3.1.2. Cell-Free Extraction Procedures

i) Triton X-100 and sonication

A wet cell weight of 20 g was suspended in sorbitol buffer A (50 ml) in a 250 ml Erlenmeyer flask. Triton X-100 solution was added to a final concentration of 0.5% v/v. The mixture was agitated on a radial shaker at 3-4°C for 30 minutes. After agitation the mixture was transferred to a 200 ml beaker (pre-cooled) immersed in crushed ice. The sonicator probe was tuned for 70W power by immersing it in distilled water and adjusting the frequency of the sonicator until the highest pitched sound was audible. The probe was then immersed in the cell suspension and the suspension was sonicated with 15 second bursts six times. A period of 60 seconds was left between each exposure to prevent heating of the suspension.

ii) Lysozyme and sonication

A wet cell weight of 20 g suspended in sorbitol buffer A (50 ml) was placed in a 250 ml Erlenmeyer flask. Lysozyme (20 mg) was added to the suspension and the mixture incubated with gentle shaking at 30°C for 12 hours. The mixture was then sonicated as described in (i) above.

iii) Lyticase and sonication

A wet cell weight of 12 g was suspended in sorbitol buffer A (50 ml) in a 250 ml Erlenmeyer flask. A lyticase solution of 100 units

per ml in 0.05 M phosphate buffer was prepared. A volume equivalent to 400 units was then added to the cell suspension and the mixture incubated at 30°C with gentle shaking for 45 minutes. The mixture was then sonicated as in (i) above.

iv) Triton X-100 and milling

A wet cell weight of 20 g was suspended in sorbitol buffer A (50 ml) and incubated with 0.5% v/v Triton X-100 as in (i) above. The mixture was then transferred to a pre-cooled Braun Homogeniser shaking bottle (75 ml) containing 0.45-0.5 mm diameter glass beads (25 g). The glass bottle was sealed and shaken at 2000 rpm for four 30 second periods, in the Braun Homogeniser. A delay of 30 seconds between each period was introduced to prevent the bottle overheating. The temperature of the glass bottle was maintained at 3-4°C by liquid carbon dioxide cooling.

v) Lysozyme and milling

A wet cell weight of 20 g was suspended in sorbitol buffer A (50 ml) and incubated with lysozyme as in procedure (ii) above. Following incubation the mixture was homogenised in a Braun Mill Homogeniser as described in procedure (iv).

vi) Lyticase and milling

A wet cell weight of 12 g suspended in sorbitol buffer A (50 ml) and incubated with lyticase as described in procedure (iii) above. After incubation the mixture was homogenised using a Braun Homogeniser as described in procedure (iv).

5.3.1.3. Removal of Cell Debris

The extracted cell suspensions were centrifuged in sterile 15 ml polypropylene tubes at 3000 g for 10 minutes. An amber coloured supernatant was designated the "cell free extract". The cell free extract was used immediately, ie without any storage, in any subsequent transformation studies.

5.3.1.4. Protein Determination

Protein determination was by the method of Sedmak and Grossberg (1977) using Coomassie Brilliant Blue G250 dye and Bovine Serum Albumin (BSA) as the standard protein. This method is thought to suffer no interference from free amino acids and chemicals such as Triton X-100 which interfere with the Lowry protein assay (Lowry *et al*, 1951). A calibration curve was constructed in the presence of the possible sources of interference such as lyticase, lysozyme codeine phosphate and Triton X-100.

A series of standard solutions of BSA in sorbitol buffer A were prepared (concentration range 10 to 100 µg/ml). The solutions also contained codeine phosphate (1.6mM),Triton X-100 (0.5v/v) and lyticase (400 units). The protein concentration was measured by adding 0.5ml of each standard solution to G250 solution in a stoppered test tube and mixing immediately. The control solution contained the sorbitol buffer A mixed with 0.5ml of G250 dye. The absorbance of each mixture was measured spectrophotometrically at

595nm against the sorbitol buffer. The absorbance of the control mixture was subtracted from the absorbance of the solutions.

Figure 5.1 shows a plot of the absorbance values of the standard solutions (minus the control) against the respective BSA concentration. The plot was subjected to linear regression analysis:

Slope = 1.632

Intercept = -2.8

Correlation coefficient = 0.997

The protein concentration in cell free extracts was calculated from equation 5.1:

$$\text{Protein Concentration} = \frac{\text{absorbance of test solution at 595nm} - \text{absorbance of control solution at 595nm}}{1.632} \quad \ldots\ldots\ldots 5.1$$

5.3.2 Analytical Methods

5.3.2.1. Detection of Codeine and Norcodeine by Reverse Phase High Performence Liquid Chromatography (HPLC)

The small volumes of the transformation mixtures made solvent extraction procedures difficult, the ion pair reverse phase HPLC method developed by Patel (1988) was used to analyse the transformation mixtures for codeine and norcodeine. The HPLC apparatus consisted of a Constametric III pump (Milton Roy, Stone, UK), a Rheodyne 7125 injection valve and a UVLC detector (Pye

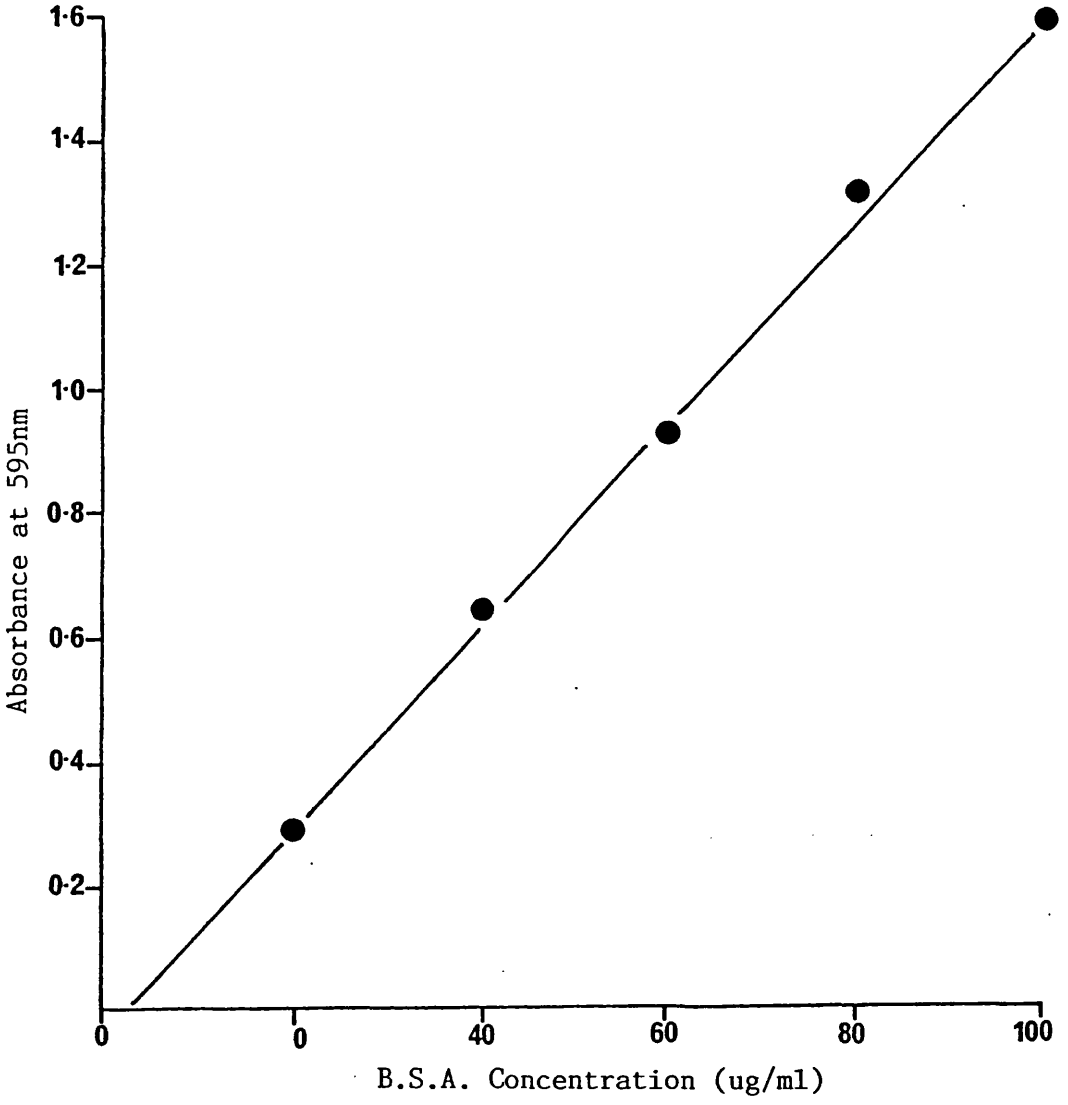


Figure 5·1 Calibration plot for protein determination using bovine serum albumin in sorbitol buffer containing codeine phosphate (1·6mM), lyticase (400 units) and triton X-100 (0·5%v/v).

Unicam). The column consisted of 250 x 4.6 i.d. column containing 6µm Spherisorb ODS stationary phase.

Column Packing

The column was slurry packed downwards in propan 2-ol using the apparatus described by Hamilton and Sewell (1982). Packing material (6µm Spherisorb ODS, 1.4g per 100mm x 5mm i.d. of column) was dispersed in about 25ml of solvent propanO-2-ol. The slurry was then poured into the slurry reservoir and attached to the guard column and analytical column (containing propan-2-ol which was displaced as the column was filled). The pump was then charged to full pressure (5000 psi), and switched on to pump the slurry through the column at full pressure. This was continued until the rate of flow of solvent from the column was constant. This was estimated by collecting the eluent over fixed time periods. At this stage the flow was switched off and the pressure allowed to fall to zero. After removing the column from the packing apparatus, a stainless steel mesh (2µm) was placed on top, followed by a porous PTFE collar on the column head.

Column Testing

The quality of the column packing was tested according to the methods of Bristow and Knox (1977). A solution containing anisole (0.03%v/v) in methanol was prepared. The mobile phase consisted of methanol and water (70:30). From the chromatogram obtained the

retention time for anisole was 2.63 min. The following equations were used to calculate the column efficiency:

$$\text{Number of theoretical plates (N)} = \frac{5.54\ t_R}{W_{0.5}} \quad \text{.....5.2}$$

Where t_R is the retention time and $W_{0.5}$ is the peak width at half the peak height.

The value of N was calculated as:

$$N = 5.54\ \frac{2.63}{3.5}^2 = 3.13$$

$$\text{The plate value (H)} = \frac{L}{N} \quad \text{.....5.3}$$

Where L is the column length and N is the number of theoretical plates. The value of H was calculated as:

$$H = \frac{250}{3.13} = 79.87$$

$$\text{The column efficiency (h)} = \frac{H}{d_p} \quad \text{.....5.4}$$

Where d_p is the particle size of the packing material and (H) is

the plate value. The column efficiency was therefore calculated as:

$$\text{Column Efficiency(h)} = \frac{79.87}{6} = \underline{13.31}$$

From Bristow and Knox (1977) this value of (h) for the 6µm spherisorb ODS column showed that the column was suitably efficient for further analytical work.

Mobile Phase

The mobile phase for the analysis of codeine and norcodeine consisted of 50% methanol and 50% 0.005M phosphate buffer, pH 6.5 containing hexane sulphonic acid (5mM). The hexane sulphonic acid was added to enhance the retention of codeine and norcodeine. The mobile phase solution was passed through a 0.45µ Millipore membrane filter under negative pressure to remove particles. The mobile phase was then degassed with helium for ten minutes prior to use.

Instrument Parameters

The instrument parameters used by Patel (1988) were used in this study for assaying codeine and norcodeine:

Flow rate	1.5ml/min	Injection volume	10µl
Temperature	25°C	Chart speed	120mm/hr
Detector wavelength	254nm		

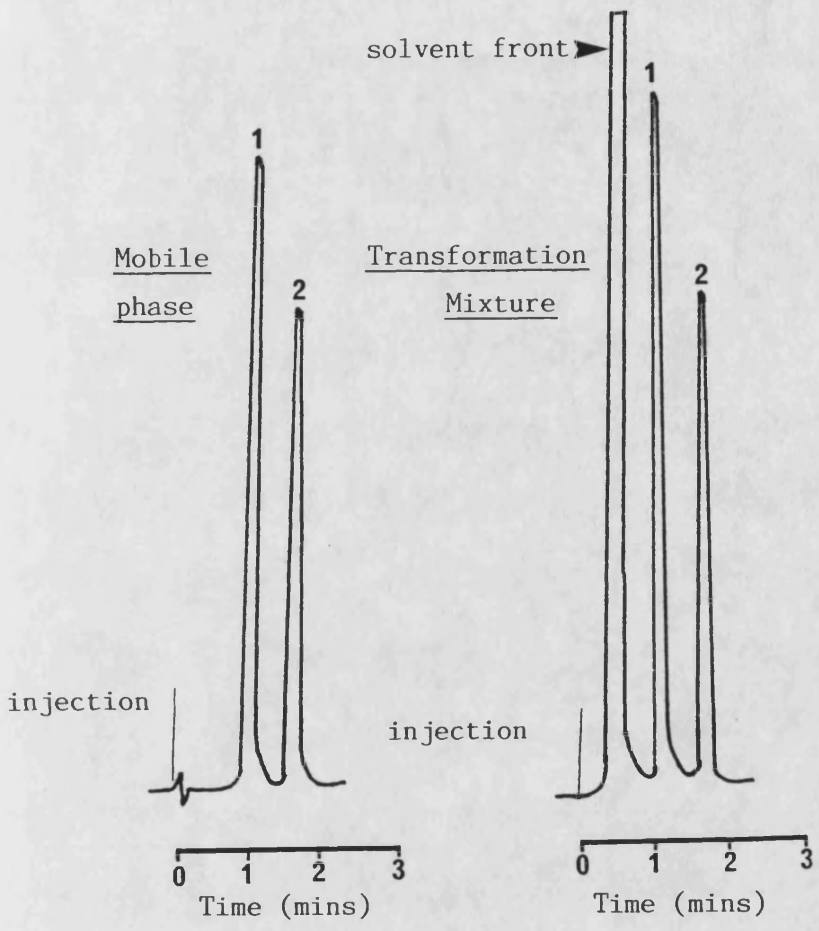
The HPLC chromatogram obtained for codeine and norcodeine in mobile phase is shown in Figure 5.2. The chromatogram is characterised by short analysis time, symmetrical peak shape and good peak resolution (separation of the peaks). HPLC analysis of codeine and norcodeine in transformation mixture also produced a chromatogram with the same characteristics (Figure 5.2)

It was therefore concluded that the instrument parameters chosen were suitable for further analysis of codeine and norcodeine.

Calibration Procedure

It was necessary to establish the suitability of the HPLC method for quantitative analysis of transformation mixtures.

Standard solutions of codeine and norcodeine were prepared containing 0.2µg/ml, 0.4µg/ml, 0.8µg/ml, 1.2µg/ml and 1.6µg/ml (of each) in mobile phase. A fixed volume (10µl) of each solution was injected 5 times and assayed as described above. The peak heights for codeine and norcodeine from the chromatograms were measured in mm. The mean of the five peak heights was calculated for each concentration of each compound. The mean peak heights were plotted against concentration of each compound and these are shown in Figure 5.3. The plots were checked for linearity by linear regression analysis and the data are shown in Table 5.1



1. Norcodeine
2. Codeine

Column: 6µm Spherisorb ODS.

Mobile phase: methanol and phosphate buffer (1:1)

Flow rate: 1·5 ml/min

Figure 5·2 Typical HPLC chromatograms for codeine and norcodeine in mobile phase and transformation mixtures.

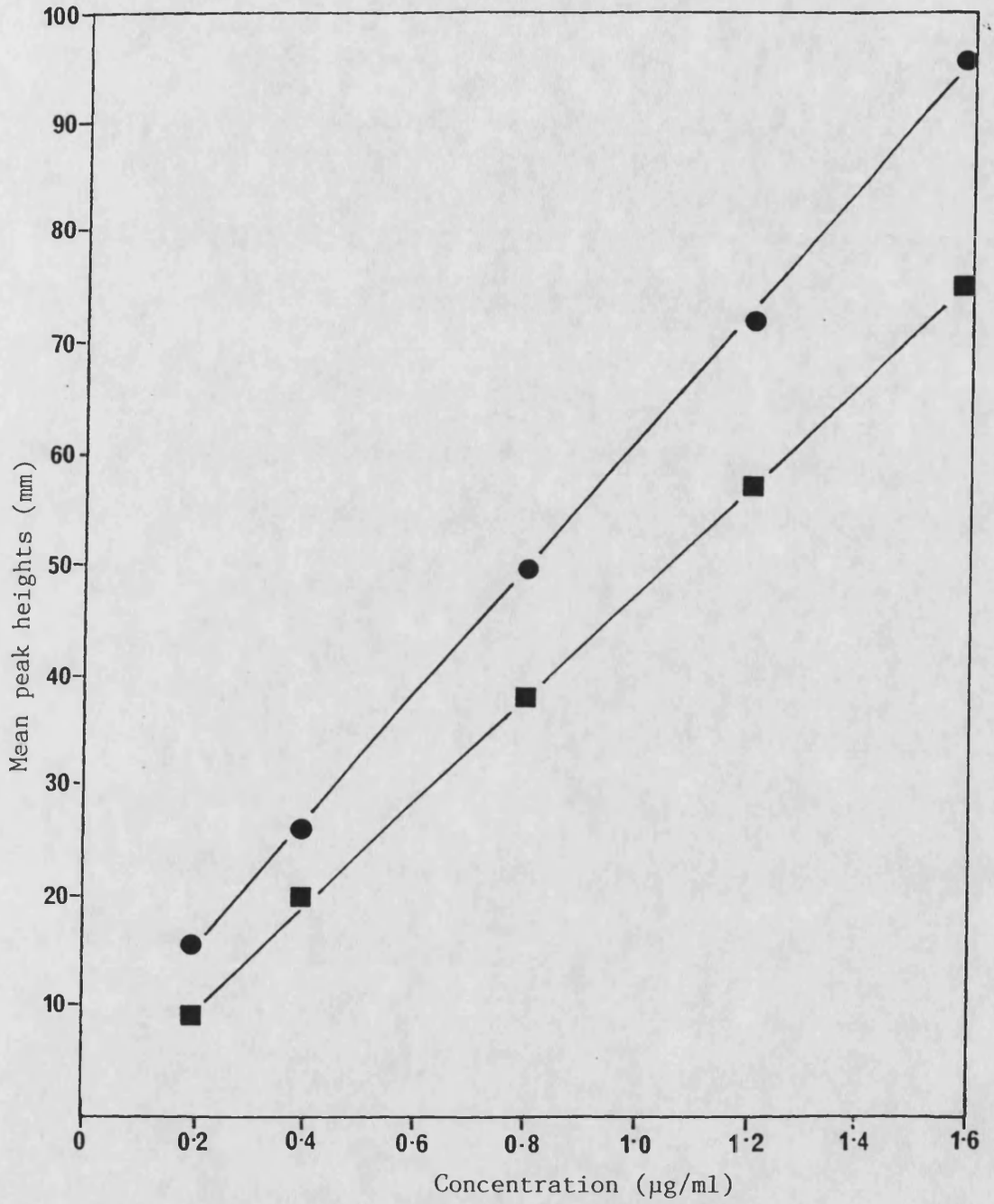


Figure 5·3 Calibration plots for reverse phase HPLC determination of codeine (●) and norcodeine (■) in mobile phase.

Value	Codeine	Norcodeine
Correlation Coefficient	0.996	0.998
Slope (S)	57.62	47.74
Intercept	3.598	-3.051

Table 5.1 Statistical data for calibration plots for reverse phase HPLC determination of codeine and norcodeine in mobile phase

The amount of codeine or norcodeine (X) in an unknown sample was determined from equation 5.4:

$$\text{Concentration (X)} = \frac{\text{Peak height mm}}{\text{Slope of plot (S)}} \quad \ldots\ldots\ldots\ldots 5.4$$

Precision of Detector Response

The precision of the detector response was determined by

preparing solutions of codeine and norcodeine (1mg/ml each) in mobile phase and transformation medium respectively. Each of the solutions was injected 10 times onto the column. The peak heights of the chromatograms obtained for codeine and norcodeine in mobile phase and transformation medium respectively were measured. The statistical analysis of the data is shown in Table 5.2

for 10 injections	Mobile Phase		Transformation Medium	
	codeine	norcodeine	codeine	norcodeine
mean peak height	92.9	76.3	93.8	74.4
standard deviation	1.152	1.31	1.01	0.88
coefficient of variation	1.73	1.87	1.82	1.91

Table 5.2 Detector response data with statistical analysis for ten injections of codeine and norcodeine in mobile phase and transformation medium.

The data showed that the detector response was reproducible for codeine and norcodeine in mobile phase and transformation medium. The precision of the detector response was also unaffected by components of the transformation medium. The detector limit was determined to be 1×10^{-3} mg/ml for codeine and norcodeine.

It was therefore concluded that the reverse phase HPLC method was sufficiently sensitive and reproducible for the quantitative

determination of codeine and norcodeine in transformation mixtures.

5.3.2.2. Determination of Codeine Transformation

The transformation mixtures after incubation were heated in a water bath at 100°C for 10 minutes to stop any reactions and transferred into polypropylene tubes, and centrifuged at 10,000g for five minutes. The supernatant was filtered through a 0.45µ cellulose acetate filter (Sartorius) and 100µl samples were assayed for codeine and norcodeine concentration using the reverse phase HPLC method described in Section 5.3.2.1.

5.3.2.3. Determination of Cytochrome P-450

Each cell free extract was subjected to the standard method of Omura and Sato (1964) to detect the presence of cytochrome P-450 as described in Section 4.3.3.1. Volumes of 2.5ml were used for each cytochrome P-450 assay.

5.4 Experimental

5.4.1 Evaluation and Development of Cytochrome P-450 Extraction Procedures

To assess the suitability of the various enzyme extraction methods described in Section 5.3.1.2., it was first necessary to

determine their efficiency under experimental conditions. The efficiency of the extraction procedures was measured in terms of the protein concentration in the resulting cell free extract, the N-demethylation activity in terms of amount of norcodeine produced from the codeine substrate and the cytochrome P-450 content of the cell free extract.

Candida tropicalis ATCC 32113 cells were grown in the fermenter as described in Chapter 4 at 1%O_2 saturation using codeine(1.6mM) as the sole carbon source. The cells were harvested and subjected to the extraction procedures described in Section 5.3.1.2. For each extraction method the weight of the wet cells used was noted and the efficiency of the extraction procedure determined. N-demethylation activity was determined using codeine as substrate in the following incubation mixture; cell free extract (5.0 ml), $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM), Semicarbazide HCl (3 mM) and 0.005 M phosphate buffer pH7 to 8 ml.

The mixtures were incubated with gentle rotary shaking (100rpm) at 30°C for 30 minutes and the reaction was initiated by the addition of codeine phosphate (1.6 mM) as substrate. The mixture was incubated for 180 minutes and the norcodeine content determined by the method described in Section 5.3.2.1. Control mixtures containing only the incubation mixture were also set up and incubated under identical conditions. The protein content of the cell free extracts was determined by the Coomassie method method in Section 5.3.1.4 and the cytochrome P-450 content by the spectroscopic method described in Section 4.3.3.2. After determining the weight of norcodeine by HPLC,

the N-demethylation activity of the cell free extract was expressed as the "total N-demethylation activity" given by equation 5.3.

$$\text{Total N-demethylation Activity} = \frac{\text{maximum norcodeine concentration } (\mu M) \times \text{total volume of mixtures(ml)}}{\text{volume of cell free extract}} \quad \ldots 5.3$$

The protein content, total N-demethylation activity and cytochrome P-450 content of each of the cell free extracts produced by methods i to iv respectively, were determined. The data obtained are shown in Table 5.3.

The results showed that highest protein yield, cytochrome P-450 content and total N-demethylation activity were obtained for cell free extracts produced using the extraction method vi. The cell free extracts produced by the other methods showed relatively low N-demethylation of codeine and low cytochrome P-450 content.

It was therefore concluded that the extraction method vi consisting of lyticase treatment followed by milling, was the most suitable method for producing cell free extracts from *Candida tropicalis* ATCC 32113 cells in subsequent experiments.

5.4.2 Characterisation of Cell Free Extracts Produced From *Candida tropicalis* ATCC 32113 Grown on Hexadecane

It was established in Chapter 4 that *Candida tropicalis* ATCC 32113 cells grown in the 7 litre fermenter on hexadecane (1%) as the sole carbon source, contained large amounts of cytochrome P-450. Because of the likely involvment of cytochrome P-450 in the N-demethylation of codeine, it was necessary to characterise the cell

Extraction Method	Cell free extract protein content mg/ml	Total N-demethylation activity uM/ml	Cytochrome P-450 nM/mg protein
i Triton X-100 & sonication	3·46	0	0
ii Lysozyme & sonication	2·1	0	0
iii Lyticase & sonication	5·8	0	0
iv Triton X-100 & milling	16·9	4·6	0·024
v Lysozyme & milling	10·3	2·1	0·021
vi Lyticase & milling	26·3	49·7	0·263

Table 5·3 N-demethylation activity, protein and cytochrome P-450 data for cell-free extracts of Candida tropicalis ATCC 32113 produced using the different methods stated.

free extracts produced from cells grown on hexadecane.

Candida tropicalis ATCC 32113 cells were grown in the fermenter using hexadecane (1%) as the sole carbon source at 1%O_2 saturation. After harvesting, the extraction method vi described in Section 5.3.1.2 was used to produce cell free extracts. The cell free extracts were analysed for protein content, total codeine N-demethylation activity and cytochrome P-450 content. N-demethylation activity was determined using the incubation mixture containing codeine(1.6mM) as described in Section 5.4.1. Control mixtures were also set up with only the cell free extract omitted, and these were incubated under the same conditions. The norcodeine content of the incubation mixtures was determined using reverse phase HPLC as described in Section 5.3.2.1. The results obtained are shown in Table 5.4.

Cell free extract	Protein mg/ml	Total N-demethylation activity (µM/ml)	Cytochrome P-450 nM/mg protein
Cells grown on 1% hexadecane	34.8	0	0.387
Cells grown on 1.1mM codeine (hexadecane grown inocula)	26.3	49.7	0.263

Table 5.4. Characterisation of cell free extracts from *Candida tropicalis* cells grown on hexadecane and codeine in the fermenter.

The data in Table 5.2 showed that cell free extracts from *Candida tropicalis* cells grown on hexadecane contained relatively large amounts of protein and cytochrome P-450 compared to cells grown on codeine. However , N-demethylation of codeine was not detected using the cell free extracts from cells grown on hexadecane. This was in contrast to cell free extracts from cells grown on codeine which showed high N-demethylation activity.

It was therefore concluded from this study that cell free extracts produced from *Candida tropicalis* cells grown on hexadecane were not suitable for further study because of their inability to N-demethylate codeine.

5.4.3. Enrichment of the Cytochrome P-450 Enzyme from Cell Free Extracts of *Candida tropicalis*

The results reported in Chapter 4 showed a correlation between cytochrome P-450 production and N-demethylation activity. To examine this correlation in more detail in cultures of *Candida tropicalis* ATCC 32113, it was necessary to attempt to purify the cytochrome P-450 present in the cell free extracts. This could be facilitated if it were possible to produce enriched microsomes from the cells.

The calcium chloride precipitation method originally used by Schenkman and Cinti (1972) and later adapted by Kappeli *et al* (1981) was used to produce cytochrome P-450 enriched microsomes from cell free extracts of *Candida tropicalis* ATCC 32113. The cells were grown in the 7 litre fermenter using codeine (1.6mM) as the sole carbon source, at 1%O_2 saturation. After harvesting, cell free extracts were

produced from the cells by extraction method vi as described in Section 5.3.1.2. The cell free extracts were then subjected to precipitation of the microsomal fraction by addition of solid calcium chloride ($CaCl_2$).

A flow diagram of the stages of the cell free extract enrichment procedure is shown in Figure 5.4. Supernatant samples were taken at each stage of the enrichment procedure and analysed for protein content, cytochrome P-450 content and codeine N-demethylation activity. The data obtained are shown in Table 5.5.

The data in Table 5.5 showed that there was a decrease in cytochrome P-450 content and protein content in supernatant samples 1 to 4 taken during the enrichment procedure. The final enriched extract, however, showed an 18 fold increase in codeine N-demethylation activity compared to the original cell free extract. The cytochrome P-450 content of the final extract was also increased to a level 33% greater than that of the cell free extract.

It was concluded that the $CaCl_2$ precipitation procedure was suitable for producing highly enriched forms of cytochrome P-450 from cell free extracts of *Candida tropicalis* ATCC 32113 whilst maintaining high N-demethylation activity. This procedure was therefore used for all subsequent experiments.

5.4.4. The Effect of Codeine Concentration on the N-demethylation of Codeine by Enriched Cell Free Extracts of *Candida tropicalis* ATCC 32113

In the transformation experiments carried out with whole cells

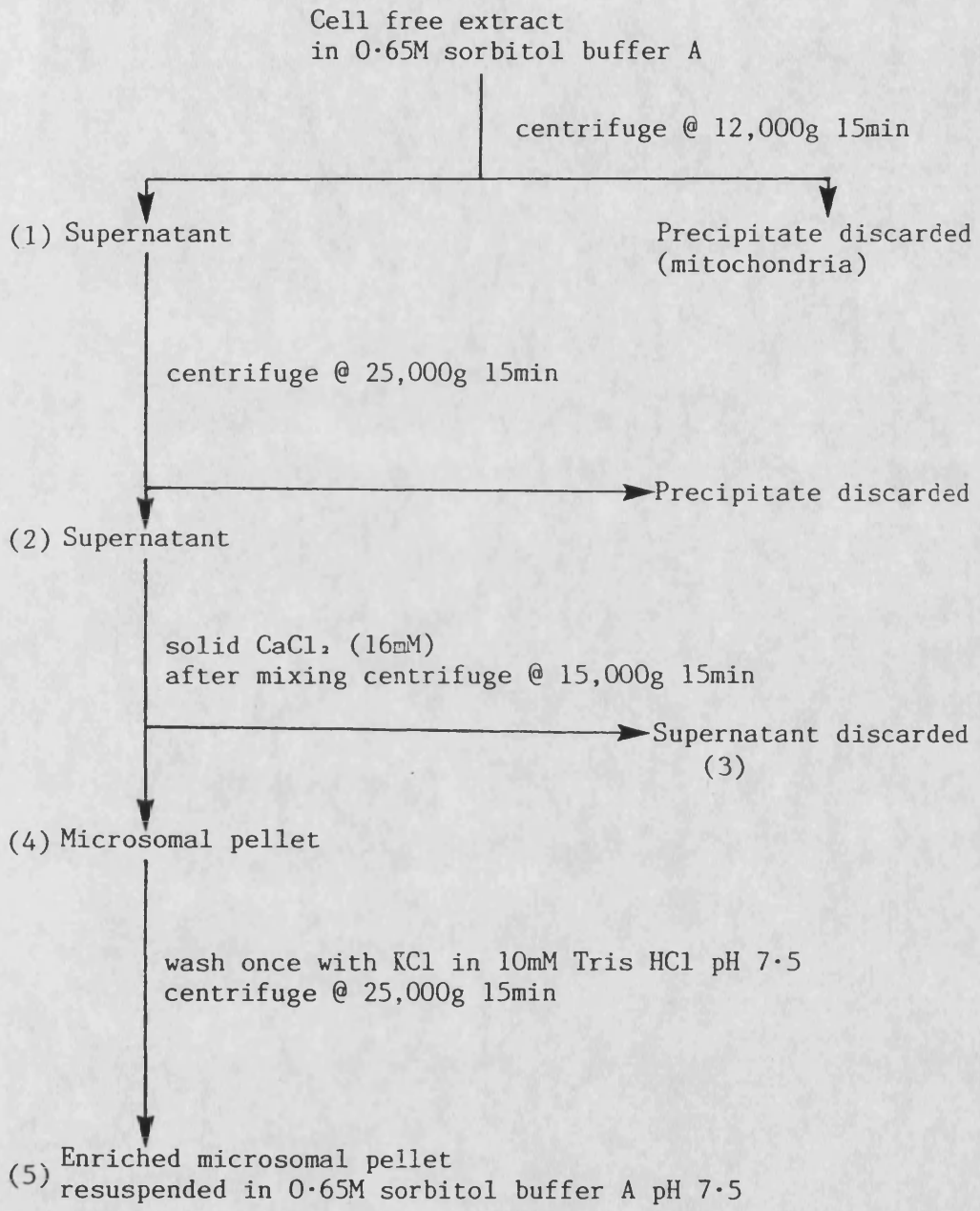


Figure 5·4 Flow diagram of enrichment procedure for cell free extracts of Candida tropicalis ATCC 32113.

Sample	Cytochrome P-450 nM/mg protein	N-demethylation activity µM/L	Protein Yield mg/ml
1	0·194	3·2	27·2
2	0·110	0·6	14·3
3	0·076	0	10·6
4	0·021	0	5·3
5 (enriched extract)	0·258	57·5	0·93

Table 5·5 Analysis of supernatant samples taken during the enrichment procedure of cell free extracts of Candida tropicalis ATCC 32113.

the maximum N-demethylation activity was observed with a codeine concentration in the medium of 1.5mM - 1.6mM. However, an optimum codeine concentration of 3 mM was used by Gibson in transformation studies using cell free extracts from *Cunninghamella bainieri*. It was therefore necessary to investigate the effect of codeine concentration on the transformation of codeine by enriched cell free extracts from *Candida tropicalis* cells.

Candida tropicalis cells were grown in the 7 litre fermenter at 1%O_2 saturation. After harvesting the cells, cell free extracts were prepared by the extraction method vi described in Section 5.3.2.1. The cell free extracts were then subjected to the enrichment procedure described in Section 5.4.3.

Transformation mixtures were prepared containing enriched cell free extract (3.0 ml), $FeSO_4.7H_2O$ (0.1 mM), NADPH (0.1 mM), NADH (0.1 mM), Semicarbazide HCl (3 mM) and 0.005 M phosphate buffer pH 7.0 to 5.0 ml. The mixtures were incubated with gentle rotary shaking (100rpm) at 30°C for 30 minutes and the reaction initiated by the addition of codeine phosphate. The concentration range of codeine used was from 1 mM to 5mM. Control mixtures were also set up containing the components of the transformation mixtures, but with the enriched cell free extracts omitted. The control mixtures were incubated under the same conditions. After 180 minutes incubation the reaction was terminated by boiling the mixtures for 10 minutes at 100°C in a water bath. The protein concentration of the total incubation mixtures was determined as described in Section 5.3.1.4 and the norcodeine content of the mixtures was determined by HPLC. The transformation activity was determined as the amount of norcodeine produced (µM) per mg of total

protein.

The plots of norcodeine produced/mg protein and protein content are shown in Figure 5.5. Norcodeine production was observed to increase with codeine concentration in the incubation mixture up to 2mM. However, concentrations of codeine higher than 2mM produced a significant decrease in N-demethylation activity. The protein content of the mixtures was observed to remain at approximately the same level at all codeine concentrations.

It was concluded that a codeine concentration of 2mM was optimal for transformation of codeine by enriched cell free extracts of *Candida tropicalis* ATCC 32113 This concentration of codeine was therefore used in all subsequent transformation experiments involving cell free extracts.

5.4.5 The Effect of Co-factors on Codeine Transformation by Enriched Cell-Free Extracts of *Candida tropicalis* ATCC 32113.

In preliminary studies with cell free extracts, the cofactors used have included NADPH, NADH and the ferrous iron (Fe^{2+}). These cofactors have been suggested to be essential requirements for microbial monooxygenase systems (Large, 1971; Ferris *et al*, 1976) as well as mammalian monooxygenase catalysed dealkylations (McMahon, 1966; Gram, 1971). These studies have reported cofactor concentrations of 0.1 mM as optimal for both microbial and mammalian systems. It was therefore necessary to establish the optimal cofactor requirements for codeine N-demethylation activity of the enriched cell free extracts of *Candida tropicalis* ATCC 32113 cells.

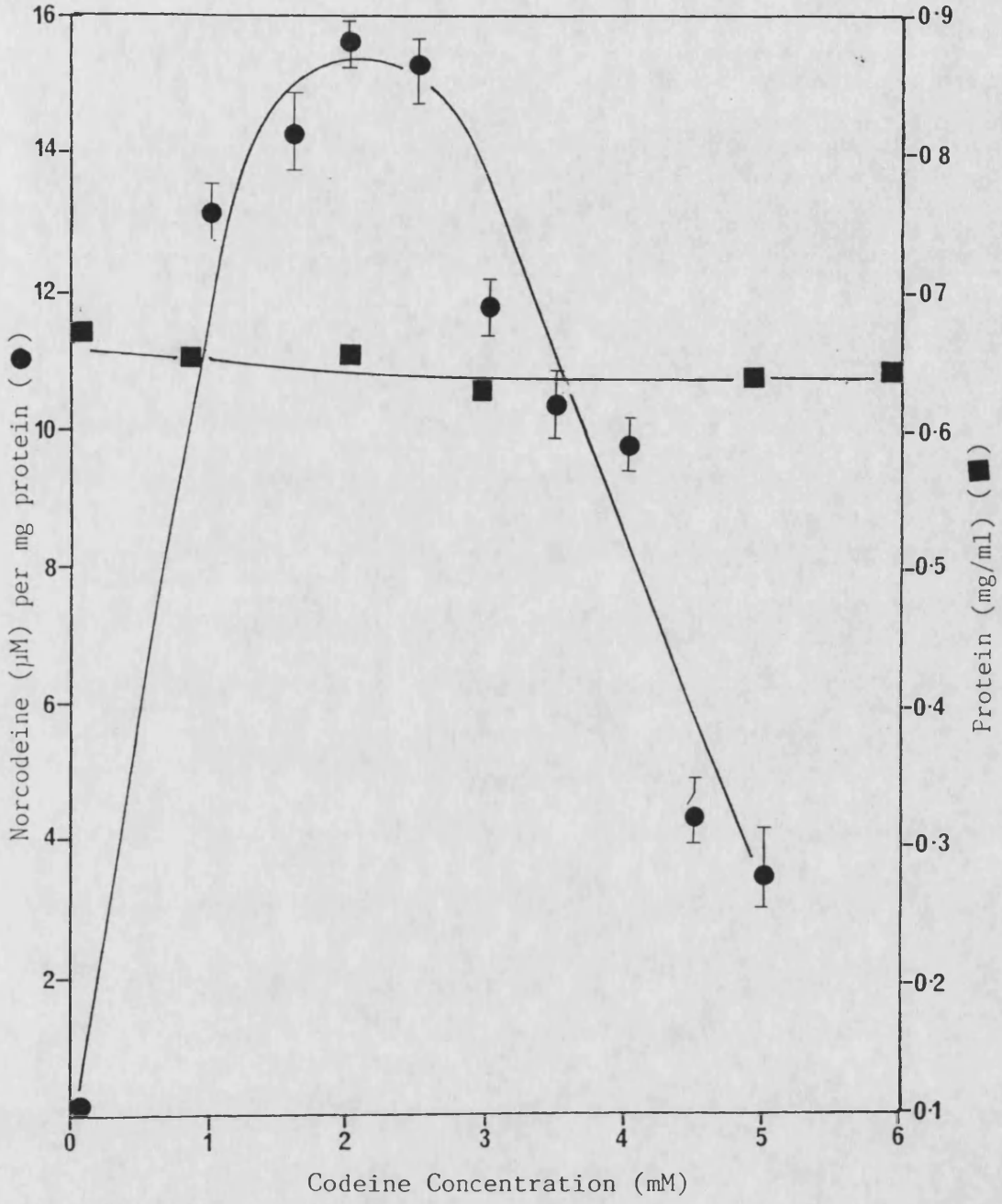


Figure 5·5 Transformation of codeine to norcodeine (●) by enriched cell-free extracts of Candida tropicalis ATCC 32113 and protein content of transformation mixtures (■) plotted against the codeine concentration in the incubation mixtures. **n = 3**

5.4.5.1. Effect of NADPH and NADH concentrations

Incubation mixtures were prepared containing the following were prepared; enriched cell free extract (3.0 ml), $FeSO_4.7H_2O$ (0.1 mM), Semicarbazide HCl (3 mM), NADPH, NADH and 0.005 M phosphate buffer(pH 7.0) to 5.0 ml. The NADPH and NADH concentrations were varied from 0 to 0.3 mM. The mixtures were incubated with gentle rotary shaking at 30°C and the reactions initiated by addition of codeine phosphate (2 mM). Control mixtures with only the NADPH and NADH omitted from the mixture were also prepared and incubated under the same conditions. After 180 minutes incubation the reactions were simultaneously terminated by boiling the mixtures for 10 minutes at 100°C in a water bath. The total protein content and norcodeine content of the mixtures was determined by HPLC. Norcodeine production was expressed as norcodeine concentration per mg protein.

The plots of norcodeine produced and protein content of mixtures against NADPH/NADH concentration are shown in Figure 5.6.

The results showed that increasing NADPH and NADH concentration from 1mM to 3mM had little influence on the norcodeine produced by the enriched cell free extracts. However the control mixtures containing no NADPH and NADH also showed relatively high norcodeine production. The protein contents of the incubation mixtures were found to be unaffected by variation in NADPH/NADH concentration.

It was concluded therefore that NADPH/NADH concentrations of 0.1mM were optimal for codeine transformation by enriched cell free extracts of *Candida tropicalis* ATCC 32113. This concentration was

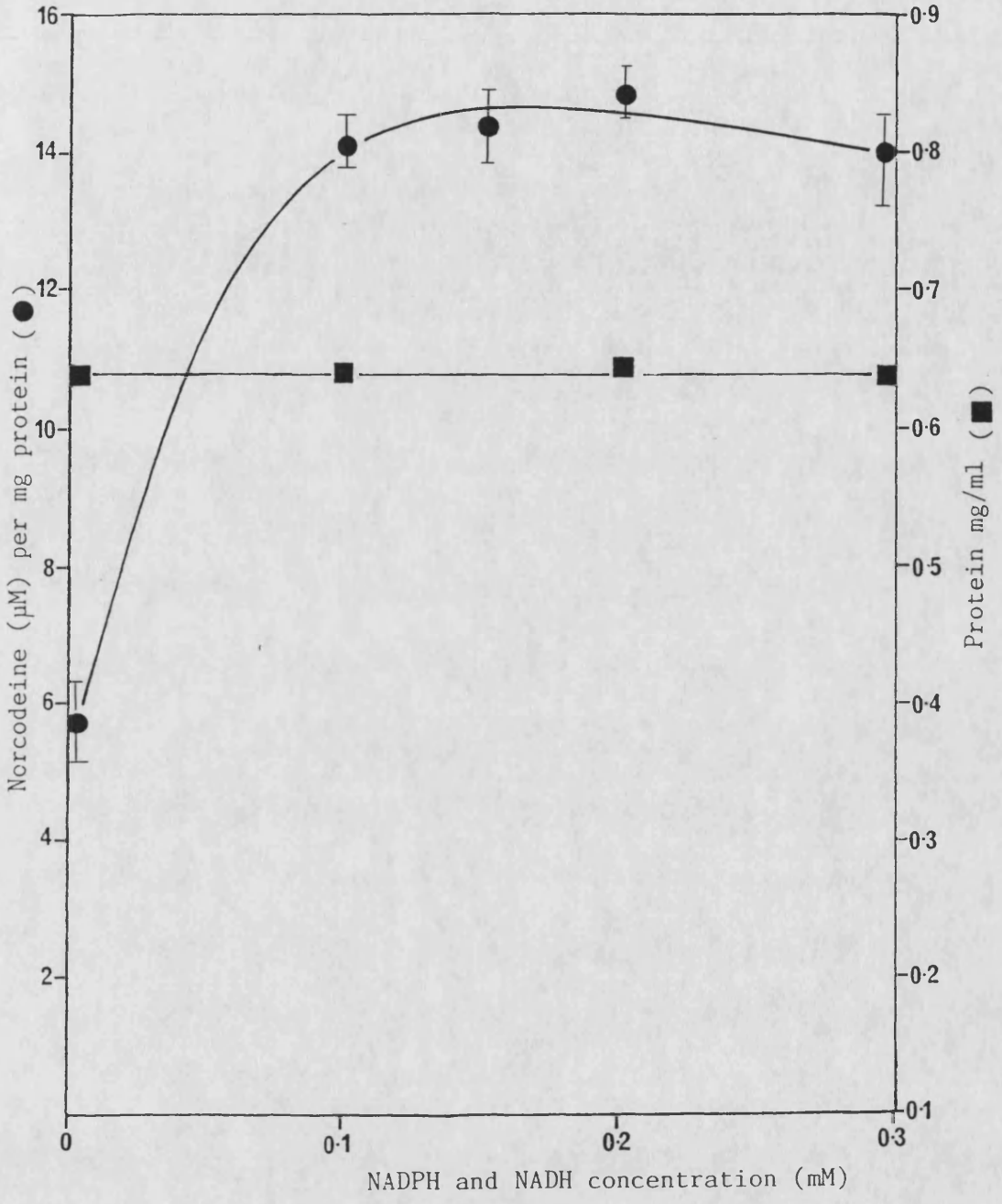


Figure 56 Transformation of codeine to norcodeine (●) by enriched cell-free extracts of Candida tropicalis ATCC 32113 and protein content of transformation mixtures (■) plotted against the NADPH and NADH concentrations in the incubation mixtures. **n = 3**

maintained for all further N-demethylation studies using enriched cell free extracts.

5.4.5.2 The Effect of Fe^{2+} Concentration

The iron molecule is part of the haemoprotien of P-450 and plays a crucial part in the activity of P-450. The effect of Fe^{2+} concentration on N-demethylation was therefore investigated. Incubation mixtures containing the following were prepared; enriched cell free extract (3.0 ml), $FeSO_4.7H_2O$, Semicarbazide HCl (3 mM), NADPH (0.1 mM), NADH (0.1 mM) and 0.005 M phosphate buffer (pH 7.0) to 5.0 ml. The ferrous iron concentration was varied from 0 to 0.3 mM. The reactions were initiated by addition of codeine phosphate (2 mM).Control mixtures with only the Fe^{2+} omitted from the mixture were also set up and incubated under the same conditions. After 180 minutes incubation and termination of the reaction by boiling, the total protein concentration and norcodeine content of the incubation mixtures were determined by HPLC.

The plots of norcodeine produced and protein content of mixtures against Fe^{2+} concentration are shown in Figure 5.7. The results showed that increasing Fe^{2+} concentration from 0.1mM to 0.3mM had little effect on norcodeine production. It was also observed that norcodeine production was not greatly reduced by the absence of Fe^{2+} in the control incubation mixtures. The Fe^{2+} concentration also had no influence on the protein contents of the incubation mixtures.

It was concluded that Fe^{2+} concentration had little effect on

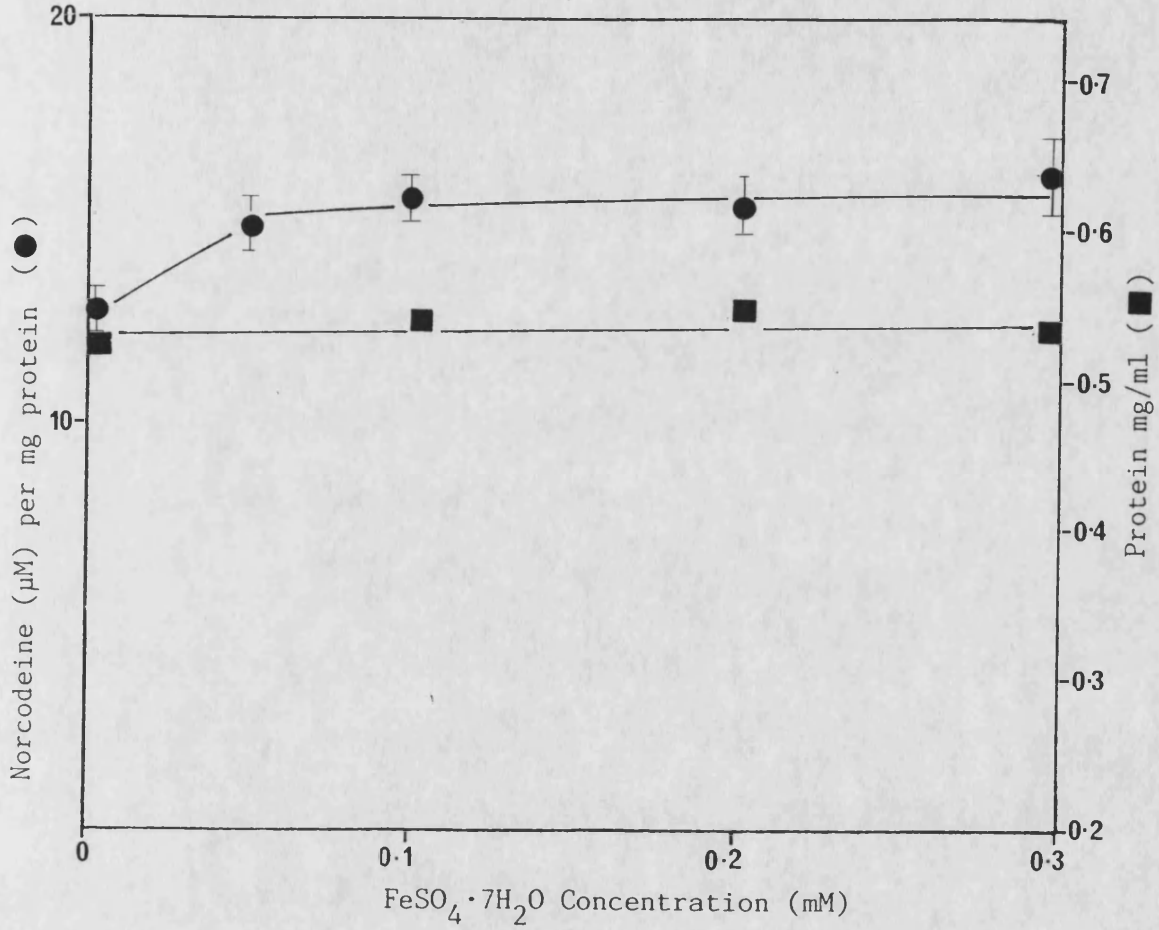


Figure 5·7 Transformation of codeine to norcodeine (●) by enriched cell-free extracts of Candida tropicalis ATCC 32113 and protein content of transformation mixtures (■) plotted against ferrous iron concentration in the incubation mixture. **n=3**

codeine transformation, therefore the concentration of 0.1mM was maintained for all subsequent N-demethylation experiments using the enriched cell free extracts.

5.4.6 The Effect of Inhibitors on N-Demethylation of Codeine by Enriched Cell Free Extracts From *Candida tropicalis* ATCC 32113

Because of the postulated involvement of cytochrome P-450 in the N-demethylation of codeine, the effect of some inhibitors of cytochrome P-450 on codeine N-demethylation was investigated.

Several cytochrome P-450 monooxygenase inhibitors have been identified and studied. Carbon monoxide and SKF-525A are known to inhibit both mammalian and microbial cytochrome P-450 systems (Adler, 1954; Gibbons *et al*, 1979; Hamill and Cooper, 1984). Potassium cyanide is known to be an N-oxide demethylase inhibitor (Large, 1971; Aoyama*et al* 1981a). Ketoconazole and miconazole are antimycotic agents and are thought to be universal cytochrome P-450 inhibitors (Borgers *et al*, 1983; Higashi *et al*, 1987).

Incubation mixtures were prepared containing the components described in Section 5.4.4. Duplicate pairs of test incubation mixtures were maintained at 3-4°C and then exposed to the following treatments:

a) Control 1 — Transformation mixture not exposed to any treatment.

by the addition of codeine phosphate to a final concentration of 2 mM. After incubation for 180 minutes, 4.0ml volumes of the mixtures were filtered through a 0.45 µm cellulose acetate membrane filter. The filtered mixtures were then assayed for norcodeine by HPLC as described in Section 5.3.2.

The effect of the presence of inhibitors on the HPLC assay for norcodeine was investigated by repeating the calibration procedure as in Section 5.3.2. with the inhibitors added at the concentrations used above. No change in the detector response to norcodeine was observed with any of the inhibitors.

The data obtained for the N-demethylation of codeine to norcodeine by enriched cell free extracts of *Candida tropicalis* in the presence of inhibitors are shown in Table 5.6. A 100% transformation was assumed for the control mixture (a) and transformations in the presence of inhibitors were calculated relative to this.

It can be seen in Table 5.6 that the N-demethylation of codeine by the enriched cell free extracts was totally inhibited by boiling and exposure to carbon monoxide. The drugs ketoconazole and miconazole and nitrogen gas caused significant but not total inhibition of the codeine N-demethylation activity of the enriched cell free extracts. Potassium cyanide, however caused only 30% inhibition of activity.

It was concluded from this study that carbon monoxide and nitrogen gases, and the drugs ketoconazole and miconazole were effective inhibitors of codeine N-demethylation activity. Since these

Inhibitor	N-demethylation Activity (% relative to control)
Control	100
Boiling	0
Carbon monoxide	0
Ketoconazole	11
Miconazole	14
Nitrogen	22
Potassium cyanide	70

Table 5·6 Effect of different inhibitors and treatments on the N-demethylation of codeine by enriched cell-free extracts of Candida tropicalis ATCC 32113

gases and drugs are considered to inhibit cytochrome P-450 monooxygenases, these data lend further support to the hypothesis that cytochrome P-450 is involved in the N-demethylation of codeine by *Candida tropicalis*.

5.4.7 Determination of Stability of Cytochrome P-450 in Enriched Cell Free Extracts

It was necessary to establish the stability data of the enriched cell free extracts during experimental procedures, and account for any loss of N-demethylation activity. Studies by Gibson suggested that activity was maintained by storage under nitrogen gas at 4°C. The results in this study (Section 5.4.6) showed there was significant loss of transformation activity when nitrogen gas was introduced into the transformation mixture.

Enriched cell free extracts, prepared from *Candida tropicalis*, ATCC 32113 were divided into 3.0 ml aliquots and sealed in 5 ml glass ampoules. Prior to sealing, a third of the ampoules were purged with oxygen free nitrogen gas for 2 minutes to displace the air and another third of the ampoules purged with oxygen gas. The remainder were sealed in air. The sealed ampoules were divided again, and some of each type were stored in a fridge at -4°C and the remainder under liquid nitrogen (-196°C) in a Union Carbide BF-6 biological freezer.

At intervals during a 72 hour storage period the N-demethylation activity ,cytochrome P-450 content and protein

content of the enriched cell free extracts was determined by the procedures described in Section 5.3.2. Freshly prepared enriched cell free extracts were used as the control and analysed as above. The values obtained for the controls were assumed to be 100% and the values of the stored extracts were calculated relative to this value.

The plots of relative N-demethylation activity against storage time are shown in Figure 5.8. Extracts stored under a nitrogen gas atmosphere showed a significant decrease in activity. The extracts stored in air and oxygen atmospheres maintained 75-80% of the original activity over the 72 hour storage period.

The plots of cytochrome P-450 contents of the extracts stored in different gaseous atmospheres at different temperatures are shown in Figure 5.9. The cytochrome P-450 contents of the extracts, stored in nitrogen gas at -4°C and -196°C, decreased to 8% of the original levels (0.021 nM/mg protein). The extracts stored under air and oxygen gas maintained cytochrome P-450 levels of 87% of the original level (0.24 nM/mg protein) at -4°C and - 196°C(Figure 5.9). The protein contents of all the extracts were observed to remain constant under all storage conditions.

On the basis of these data enriched cell free extracts from *Candida tropicalis* ATCC 32113 were stored in air at -4°C and used within 48 hours of storing. Under these storage conditions the codeine N-demethylation activity and cytochrome P-450 content of the enriched cell free extracts were preserved at acceptable levels.

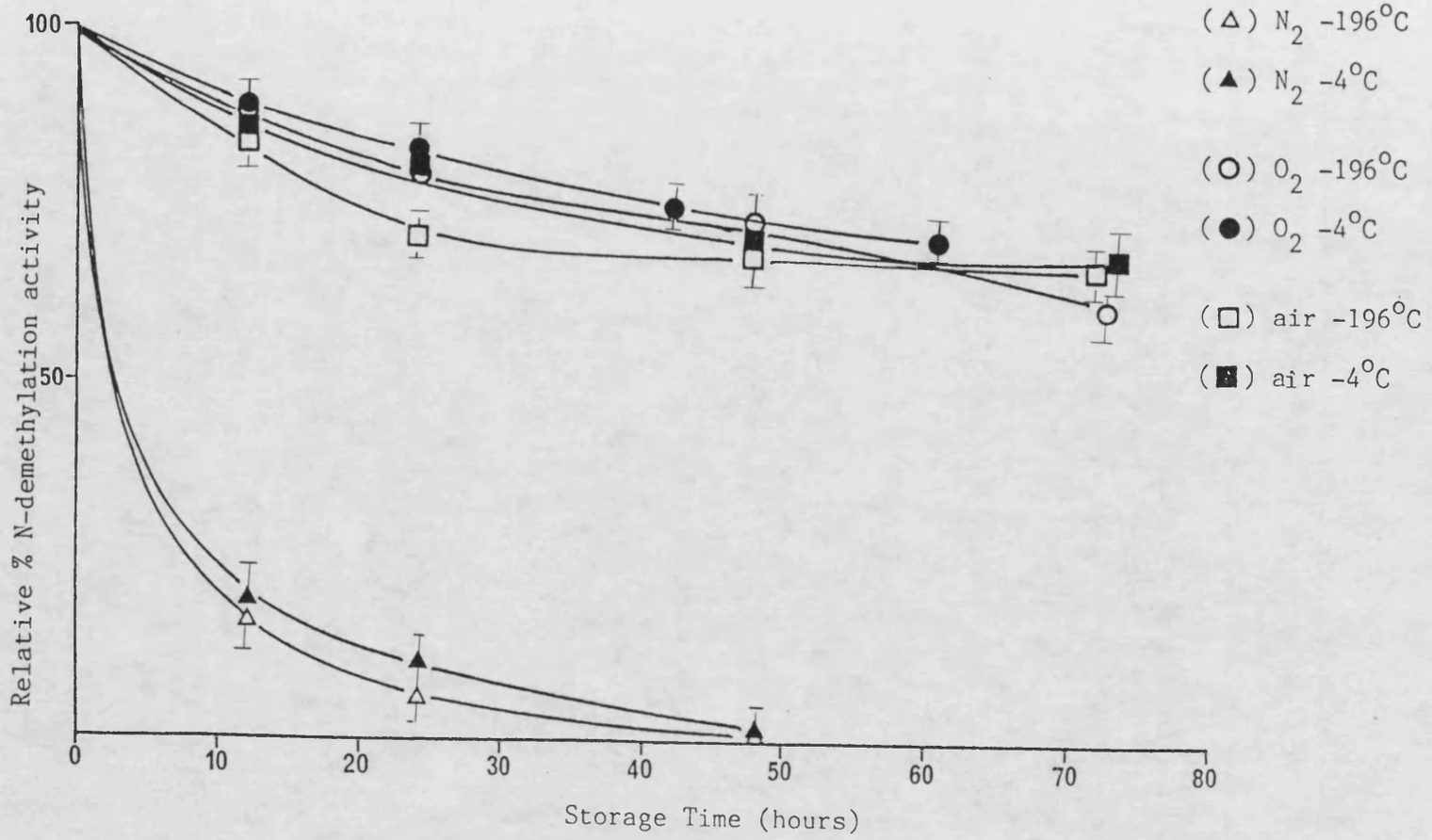


Figure 5·8 The effect of storage under different conditions on the transformation of codeine by enriched cell-free extracts of Candida tropicalis ATCC 32113. **n = 3**

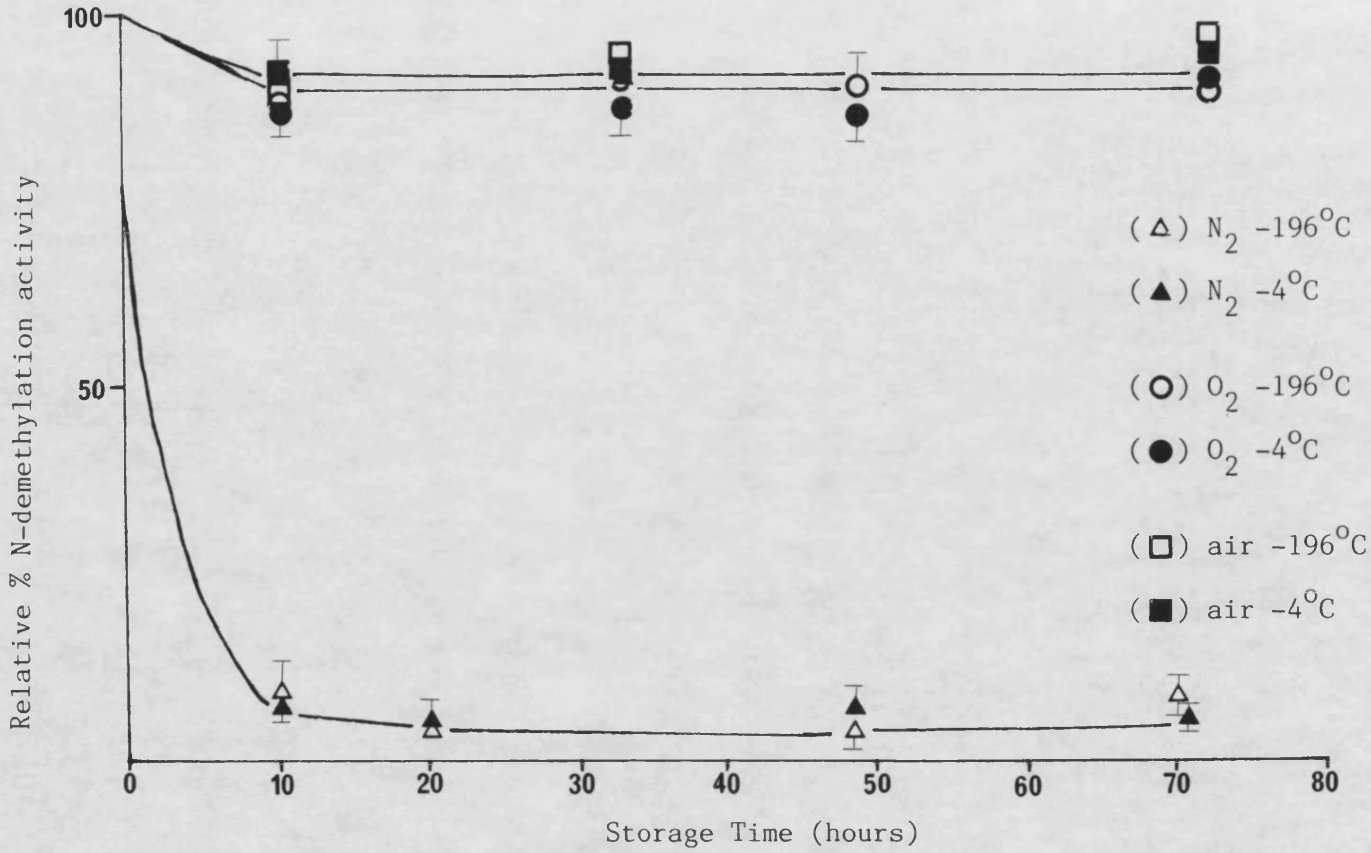


Figure 5·9 The effect of storage under different conditions on the transformation of codeine by enriched cell-free extracts of Candida tropicalis ATCC 32113 **n=3**

5.5 Discussion

In order to characterise the cytochrome P-450 monooxygenase system in *Candida tropicalis*, it was necessary to find a suitable cell disruption and extraction method. The methods, however, also needed to produce no interference with the protein determination assay. The Lowry protein assay (Lowry *et al*, 1951) has been used before (Sewell, 1982) but is known to suffer interference from the non-ionic surfactant Triton X-100. The Coomassie Blue protein assay (Sedmak and Grossberg, 1977) was therefore used. The extraction methods surveyed did not interfere with this assay.

Triton X-100 has been commonly used for solubilizing membrane bound enzymes (Ito and Sato, 1968; Spatz, 1971) and its mode of action described (Cheetham *et al*, 1980). Triton X-100, however, proved very unsuccessful in the extraction procedures and only a small degree of N-demethylation activity was observed when it was used in conjunction with ballmill homogenisation. Pringle (1978) has postulated that Triton X-100 is unable to penetrate the cell wall of yeast cells due to an uptake-exclusion threshold of the cell wall corresponding to a polydisperse polyethylene glycol of average molecular weight 650. A value of 620 has also been estimated by Scherrer *et al*, 1974 for bakers' yeast. Since the molecular weight of Triton X-100 has been postulated to be 628 (Inque and Kitagawa, 1976) it may be assumed that the surfactant was excluded from entering the cell wall. The Triton X-100 may have also interacted

with the cytochrome P-450 after the ballmill procedure and caused denaturation of the enzyme. This effect of Triton X-100 has been observed by Yoshida and Kumaoka, 1964. The cell wall lysing enzyme lysozyme (Chipman and Sharon, 1969) was also unsuccessful at breaking down the cell wall and N-demethylation activity was only detected after ballmill homogenisation.

Lyticase, from *Arthrobacter luteus*, is a yeast cell wall lysing enzyme and acts by breaking down the glucan in the yeast cell wall (Scott and Schekman, 1980; Mitaszko *et al*, 1981). Microscopical examination revealed only lyticase treatment caused conversion of the *Candida tropicalis* cells into spheroplasts. Spheroplasts were characterised as yeast cells having a bulging spherical shape caused by breakdown of the cell wall. Sonication following lyticase treatment failed to disrupt the cells, hence no activity was detected in the resulting cell free extract. These studies suggested that sonication was incapable of breaking up *Candida tropicalis* cells after enzyme treatment. This is in contrast to studies by Kappeli *et al*, 1982 where yeast cells were readily lysed by sonication after zymolyase treatment.

Ballmill homogenisation after lyticase treatment, in this study, produced the highest protein concentration, N-demethylation activity and cytochrome P-450 concentrations. Hitchcock *et al* (1989) have sugggested that the N-demethylase enzyme *Candida albicans* is sensitive to increases in temperature during cell breakage and proposed ballmill homogenisation as the most suitable method for cytochrome p-450 extraction from *Candida* sp. Yeast cells have previously been commonly disrupted using cold French pressure cells

(Duppel *et al*, 1973; Aoyama *et al*, 1981a), however ballmill homogenisation is also now used (Gmunder *et al*, 1981b; Sanglard *et al*, 1987).

The HPLC assay developed for cell free extract demethylation activity provided important advantages over GLC and TLC:

1) Any level of O-demethylation activity would have been detected by the presence of morphine in the samples, because the HPLC assay was capable of detecting morphine. The absence of morphine in the experimental mixtures suggested only N-demethylation activity was occurring.
2) Whole samples were readily analysed without the need for solvent extraction procedures. Therefore the total norcodeine concentrations in the mixtures was determined.
3) The presence of the incubation mixture components such as the cofactors did not interfere with the assay. HPLC is now extensively used in drug transformation studies (Kunz *et al*, 1985). The use of control mixtures where codeine and/or enriched cell free extracts were omitted provided evidence that norcodeine formation was only due to transformation by the cell free extracts.

The absence of codeine N-demethylation activity of the cytochrome P-450 from hexadecane grown cells suggests hexadecane was inducing a different type of cytochrome P-450 from the codeine grown cells. It was however essential for a hexadecane grown inoculum to be used in experiments where codeine was the sole carbon source. This was evident as no growth was detected in cultures using codeine grown inoculum. This suggests that hexadecane grown cells were able to

adapt to assimilate codeine and use this as a sole carbon source. The codeine may then be inducing a type of cytochrome P-450 specific for the N-demethylation of codeine. The induction of specific types of cytochrome P-450 in *Candida tropicalis* has been observed by Duppel *et al* (1973) where tetradecane induces a cytochrome P-450 which specifically catalyses the hydroxylation of fatty acids.

In the attempt to purify the cytochrome P-450 in the cell free extracts the dialysis method used by Gibson, 1984 was not attempted due to the significant reduction in N-demethylation activity noted. The method of Kappeli *et al*,(1982) however was adapted to produce cytochrome P-450 enriched cell free extracts by precipitation of the microsomal fraction by solid $CaCl_2$. The enriched extracts although having a low protein yield retained a high degree of N-demethylation activity and cytochrome P-450 content. The procedure produced only small quantities of enriched cell free extract but the conveniences of the procedure and the purity of the cell free extracts obtained warranted its continued use. A procedure to purify cytochrome P-450 from cell free extracts of *Pseudomonas putida* has been described by Gunsalus and Wagner, (1955). The procedure however was very time consuming requiring ammonium sulphate fractionation, gel filtration and cellulose chromatography and a starting wet weight in kilograms was required to produce a small quantity of purified cytochrome P-450. The method was therefore not suitable for this study.

The N-demethylation of codeine by enriched cell free extracts was observed to increase up to substrate concentrations of 2 mM in the incubation mixture (Figure 5.4). There was a significant fall in

activity at concentrations above 3 mM suggesting catabolite repression of the cytochrome P-450 enzyme. This has been previously suggested by Elison and Elliott (1964) where the demethylation of codeine by rat microsomal preparations was inhibited by high codeine concentrations. In the absence of Fe^{2+}, NADPH and NADH N-demethylation activity was present in the enriched cell free extracts (Figures 5.6 and 5.7), but increasing concentrations up to 0.3 mM did not increase activity further. The activity in the absence of added cofactors suggests that an endogenous supply of cofactors was available. The cofactors are thought to be essential in cytochrome P-450 monooxygenase reactions (Dawson and Eble, 1986; Murray *et al*, 1985). The ferrous iron is thought to mediate reduction of molecular oxygen in the cytochrome P-450 substrate complex and therefore permit incorporation of an oxygen atom into the substrate (Black, 1987). NADPH and NADH are thought to serve as physiological electron donors in the cytochrome P-450 linked monooxygenases (Aoyama *et al*, 1978; Mohr *et al*, 1981; Sato and Omura, 1978) to further facilitate the incorporation of the oxygen atom in the substrate. The availability of cofactors during the reaction may have been one of the rate limiting factors in the N-demethylation reaction. This has been postulated by Lu *et al*,(1984) in the O-deethylation of 7-ethoxycoumarin. Therefore endogenous co-factors present in the cell free extract were utilized in the reaction and were not replenished. Because the identity of the endogenous cofactors was not known, it was not possible to add them to the incubation mixture to further improve the transformation yield.

In the inhibitor studies boiling the enriched extracts and carbon monoxide gas completely inhibited N-demethylation activity, whereas nitrogen gas produced 22% inhibition. Boiling of the extracts suggests complete denaturation of the cytochrome P-450. For cytochrome P-450 monooxygenases to function in N-demethylation reactions, it is necessary for oxygen to be incorporated into the enzyme-substrate complex (Kedderis *et al*, 1983). The carbon monoxide and nitrogen gases may have caused inhibition of the N-demethylation reaction by forming inert complexes with the monooxygenase system thereby preventing electron transfer and oxygen incorporation into the substrate. This effect has been observed by Hamill *et al* (1984) but reversal of the CO inhibition of N-demethlation of N'N'-dimethylaniline was noted on re-exposure to oxygen gas using hepatic microsomes containing cytochrome P-450. No such reversal was observed in this study by re-exposure to oxygen of enriched cell free extracts initially exposed to nitrogen and CO gases.

Potassium cyanide produced 30% inhibition of codeine N-demethylation. Potassium cyanide is known to be an N-oxide demethylase inhibitor. The possible mechanisms for the oxidative N-dealkylation of substrates such as codeine containing tertary amine functions by both mammalian and microbial enzyme systems has been widely debated mainly because the reaction intermediates are transient and have not been observed. Two routes of tertiary amine oxidation have been proposed for this process (Rose and Castagnoli, 1983). These are illustrated in Figure 5.10. The first primary oxidative attack is thought to occur upon the carbon to the nitrogen (α-C oxidation) to give a carbinolamine. N-dealkylation

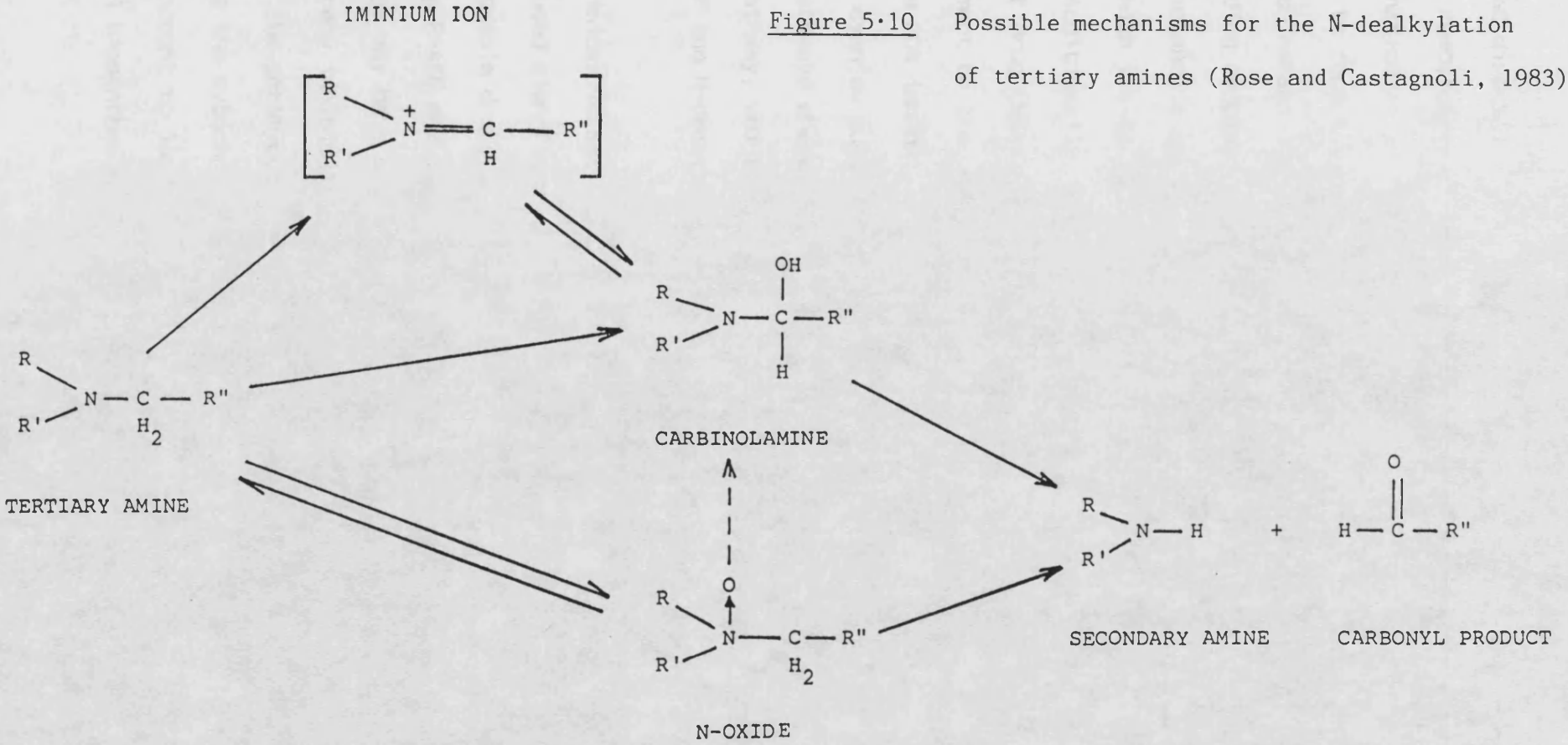


Figure 5·10 Possible mechanisms for the N-dealkylation of tertiary amines (Rose and Castagnoli, 1983)

may proceed directly *via* carbinolamine formation by an oxene insertion reaction or alternatively the substrate may undergo dehydrogenation to an imminium ion which then adds the equivalent of hydroxide to form the carbinolamine. The carbinolamine then cleaves due to its instability and form the secondary amine and the corresponding carbonyl product (Figure 5.10). The second mechanism proposes metabolic attack on the nitrogen (N-oxidation) to produce an N-oxide which yields the coresponding secondary amine and carbonyl product. Additionally tertiary amine N-oxides are readily reduced to the parent drug (Hlavica *et al* 1978) and some may undergo rearrangement to the corresponding x-carbinolamine (Gorrod *et al* 1975). The 30% inhibition of codeine N-demethylation observed using potassium cyanide suggests that the N-demethylation may be proceeding by both pathways where potassium cyanide may have inhibited the N-oxide pathway, while leaving the carbinolamine pathway to produce the 70% of the N-demethylation activity.

The imidazole antimycotic agents, ketoconazole and miconazole also produced significant inhibition of N-demethylation activity. These imidazole drugs are thought to be universal inhibitors of cytochrome P-450 enzymes (Nigai *et al*, 1986; Higashi *et al*, 1987). These drugs may have specifically interacted with the cytochrome P-450 thereby inhibiting the N-demethylation reaction. The clinical action of the antimycotic drugs is thought to occur by specifically inhibiting the cytochrome P-450 enzymes in the yeast cell membrane. This is thought to lead to weakening of the yeast cell wall due to ergosterol biosynthesis inhibition (Van den Bossche *et al*, 1980;

Loose *et al*, 1983; Meredith *et al*, 1985).

Storage of the enriched cell free extracts under nitrogen gas dramatically reduced N-demethylation activity and P-450 content. Extracts stored under oxygen and air however retained a high degree of N-demethylation activity and P-450 content. This again suggests that nitrogen gas may have been forming an inert complex with the cytochrome P-450 thereby rendering it inactive. The extracts however retained N-demethylation activity when stored under air. This was unexpected since the major component of air is nitrogen. This suggests that the oxygen in the air may have been protecting the cytochrome P-450 active site from the irrevrsible interaction with nitrogen. The interaction with nitrogen is irreversible because bubbling oxygen into the cell free extracts stored in nitrogen did not return the N-demethylation activity. For convenience, extracts were stored in air at -196°C and used within 48 hours.

CHAPTER SIX

CONCLUDING DISCUSSION

CHAPTER 6. CONCLUDING DISCUSSION

N-demethylation reactions are often required and used in the synthesis of drug intermediates, but the chemical methods to achieve the reaction often prove difficult. In an attempt to find alternatives, previous studies at the University of Bath demonstrated the ability of the fungus *Cunninghamella* species to N-demethylate codeine and other drug molecules containing the N-methyl function (Sewell, 1982; Gibson, 1984). However several disadvantages were encountered with the use of *Cunninghamella* sp. for these studies. The organisms were slow growing and codeine transformation was therefore time consuming. Furthermore, the filamentous nature of the fungus made quantitative growth determination difficult. Problems of surface attachment were also encountered when attempts were made to scale up growth using fermenters.

It was the aim of this study to develop an alternative microbial transformation system, using *Candida* sp. , that was capable of effecting N-demethylation of drug molecules. The ability of *Candida* sp. to oxidise higher aliphatic hydrocarbons to their hydroxy derivatives, has been demonstrated (Lebeault *et al*, 1971). The opiate alkaloid codeine was selected in this study as the test substrate because of its capacity to be both N- and O-demethylated. Codeine was also used as the test substrate in the previous studies using *Cunninghamella* sp. (Sewell, 1982: Gibson, 1984).

Although the present study was mainly concerned with the development of an alternative transformation system to that of *Cunninghamella* sp , only a small number of *Candida tropicalis* strains were examined and screened for N-demethylation activity. It could therefore be beneficial to extend the screening studies to include other strains of *Candida tropicalis* and other *Candida* sp. Studies by Hitchcock *et al* (1989) have demonstrated the ability of *Candida albicans* to N-demethylate lanosterol by a cytochrome P-450 enzyme. This could be expanded to investigate the ability of *Candida albicans* to N-demethylate codeine and other drug molecules. Other species of yeasts, including *Saccharomyces* sp. could also be examined since some species of *Saccharomyces cerevisae* have been shown to possess N-demethylation activity (Aoyama *et al*, 1981a).

The initial experiments in this study established methods for accurate growth determination of *Candida tropicalis*. The surface spread method provided the most accurate determination of the number of viable cells during growth. The use of optical density measurements could not provide an accurate determination of the number of viable cells. It was however useful for rapid determination of the number of cells. Determination of viable count by the surface spread method was a time consuming procedure and it may be beneficial to investigate other methods for viable count determination. The initial lag and exponential phases of *Candida tropicalis* cells grown on codeine as a sole carbon source were different from cells grown on glucose. The codeine grown cells showed a longer lag phase but the same exponential incline. Further experiments could be

carried out to investigate the effect of secondary cultures grown on codeine, as inocula, on the initial lag phase of growth.

The solvent extraction method used prior to GLC analysis established 90% efficiency for extracting possible transformation products from the cultures. Further organic solvents or mixture of solvents should however be investigated in order to increase extraction efficiency even further. The use of the OV17 column in GLC analysis showed that derivitisation of the samples was unneccessary to achieve resolution of the possible transformation products. This was an advantageous step forward over the GLC procedures used by Sewell (1982) and Aoyama *et al* (1981a) where derivitisation of the extracted samples was necessary before GLC analysis, leading to increased time of experimental procedure. The TLC method provided rapid analysis of the transformation mixtures but suffered from low sensitivity in detecting the possible products. It may be possible to develop a TLC system with increased sensitivity to codeine, norcodeine and morphine where this would provide rapid analysis of transformation mixtures prior to extraction procedures. It was observed that upto two minor spots appeared for all standard solutions and extracted samples analysed. Using preparative TLC on glass plates, the minor spots could be extracted and analysed for identification and further experiments carried out as to their effect, if any, on codeine tranformation by *Candida tropicalis*. This procedure has been carried out by Aoyama *et al* (1981) where the products of lanosterol N-demethylation by *Saccharomyces cerevisae* were separated by TLC on silica gel plates and subsequently scraped off and analysed by GLC.

The sreening studies showed that *Candida tropicalis* strains NCYC 997, ATCC 22577 and 32113 demonstrated the ability to demethylate codeine. A mixture of codeine N- and O-demethylation activity was confirmed for the *Candida tropicalis* strains NCYC 997 and ATCC 22577. This was apparent because of the appearance of both morphine and norcodeine peaks on GLC analysis of the cultures. This suggests that codeine was inducing either a specific type of cytochrome P-450 capable of both N- and O-demethylation or that two different types of cytochrome P-450's were being induced by codeine. Further experiments using controlled conditions in a fermenter could be carried out to investigate the effect of culture conditions such as oxygen availability on the specificity of the cytochrome P-450 produced. In the present study codeine N-demethylation activity only was observed with *Candida tropicalis* ATCC 32113 and this strain was therefore used for further investigation. In the growth medium development studies, the composition of the chemically defined growth medium was shown to affect growth of *Candida tropicalis* cells and N-demethylation of codeine. The use of the modified medium of Hug *et al* (1974) (Medium B) optimised both growth of the *Candida tropicalis* cells and codeine N-demethylation. This suggests that some components of the Hug medium had a large influence on the *Candida tropicalis* cells. Further experiments could be carried out to identify the key components of the Hug medium affecting growth. The use of hexadecane grown inocula was shown to influence codeine N-demethylation. The primary cultures grown on hexadecane showed prolific growth and these were subsequently used as inocula. Codeine N-demethylation activity of

Candida tropicalis ATCC 32113 was shown to increase by a factor of 3 using inocula grown on hexadecane. N-demethylation activity and cell growth was shown to be reduced using inocula grown on glucose and codeine. This suggested that hexadecane may have been inducing a specific type of cytochrome P-450 which was readily able to utilise codeine as a carbon source whereas glucose may have been inducing another type of cytochrome P-450 unable to use codeine. Glucose has been shown to induce a "different" type of cytochrome P-450 in *Candida tropicalis* and *Saccharomyces uvarium* by Sanglard *et al* (1984) where the cytochrome P-450 was specific for the 14α-demethylation of lanosterol. Further experiments should be carried out to investigate the substrate specificities of different *Candida* sp grown on glucose. The substrate specificity induced by hexadecane on *Candida tropicalis* ATCC 32113 suggests that the mechanism of codeine uptake may be similar to hexadecane uptake by *Candida* cells. Gmunder *et al* (1981) have suggested that hexadecane is taken up by *Candida* cells by a combination of pinocytosis and simple diffusion. The effect of other potential inducers of cytochrome P-450 and codeine N-demethylation needs to be investigated. The study may be continued by screening a wide range of n-alkanes for cytochrome P-450 induction capability. Studies by Sanglard *et al* (1984) have used a mixture of n-alkanes to induce the catalytic activity of cytochrome P-450 in yeast cells. The use of n-alkane mixtures may be beneficial in the induction of N-demethylation activity in *Candida tropicalis* cells. ^{13}C NMR spectroscopy has been used to detect possible intermediates in the N-demethylation of codeine (Gibson 1984). NMR spectroscopy was not employed in this study, but could be used together with other drug

molecules containing ^{13}C enriched N-methyl groups or ^{13}C enriched in the nucleus to further characterise the N-demethylation reaction. It may also be possible to identify the exact site of N-demethylation in the microbial cell with the aid of radiolabelled precursors.

The volume of inoculum used was shown not to influence N-demethylation activity or growth of *Candida tropicalis* cells. By increasing the codeine concentration above 2mM in the transformation cultures, the N-demethylation activity was dramatically reduced. This observation was attributed to substrate inhibition of the transformation system. This has been observed in mammalian metabolic studies using codeine (Elison and Elliott, 1964). The use of casein hydrolysate as the sole nitrogen source inhibited growth and codeine N-demethylation activity of the *Candida tropicalis* cells. Decrease in growth and N-demethylation activity was also observed when concentrations of the vitamins was doubled and when folic acid was added to the medium. These effects have been observed by other workers who have used vitamins in their medium (Gmunder *et al* 1981). Vitamin concentration was therefore not altered and folic acid was not added to the medium B in subsequent experiments. The transformation of codeine to norcodeine by *Candida tropicalis* ATCC 32113 was relatively low with percentage conversion yields of between 0.6 and 1.1%. However a defined, reproducible transformation system was established for *Candida tropicalis* ATCC 32113 and this provided a basis for further scale up fermenter studies.

The use of the 7 liter fermenter provided a method for studying

the transformation system using *Candida tropicalis* under very controlled conditions. Cytochrome P-450 has been postulated as the enzyme responsible in the *Candida tropicalis* cells for the N-demethylation of codeine. The involvement of cytochrome P-450 in N-demethylation reactions has been demonstrated by Aoyama *et al*,(1986). Codeine N-demethylation activity and the P-450 content of cells was highly influenced by $\%O_2$ levels in the medium. A 1% O_2 saturation level was observed to optimise N-demethylation activity and P-450 content of cells. This was supportive of the hypothesis that low $\%O_2$ was inducing P-450 production in *Candida tropicalis* cells and consequently increasing codeine N-demethylation activity by the cells. The use of hexadecane at low $\%O_2$ levels was found to increase growth rate of the cells and further induce P-450 production by the cells. Increases of cytochrome P-450 concentration in *Candida* yeasts by oxygen limitation has so far only been demonstrated for growth on n-alkanes. This may be further investigated by conducting experiments with *Candida* at low oxygen concentrations using other substrates. A possible explanation of the oxygen effect from the physiological point of view could be the following; the low $\%O_2$ in the medium influences the hydroxylation rate of the alkane hydroxylating monooxygenase system which contains the cytochrome P-450. The *Candida* cell subsequently responds to the decreased hydroxylation rate with an increase in the biosynthesis of the components of the monooxygenase system including cytochrome P-450. The increase in growth rate observed at low $\%O_2$ may be explained by the other metabolic processes essential for growth overcompensating for the low $\%O_2$. Continuos culture experiments with *Candida*

tropicalis by Gmunder *et al* (1981) have also shown that oxygen limitation increases cytochrome P-450 production using n-alkanes. Codeine N-demethyaltion activity and P-450 content of cells was also optimised at culture pH levels of 5.5. This suggests that the *Candida tropicalis* cells may have been taking up codeine in the ionised form by a pinocytosis/diffusion process similar to that for hexadecane.

A stirrer speed of 550 rpm was found to be sufficient to achieve maximal growth and P-450 production by the cells. Erickson and Nakahara (1975) however have demonstrated that the length of the exponential growth phase of *Candida* cells is related to the degree of dispersion and correlated to the stirring intensity. Therefore further experiments could be carried out to study the relationship between stirring intensities and the exponential growth phase of *Candida tropicalis* strains grown in continuous culture. In future experiments a continuous culture method would be advantagous because with batch cultures it is difficult to determine harvest time at maximum cellular cytochrome P-450 content. The optimum concentration of P-450 is obtained in a relatively narrow time interval and batch cultures are not highly reproducible. During continuous culture the influence of individual parameters can be best evaluated and the optimum conditions can be maintained independently of time (Trinn *et al*, 1982). Therefore identical cell material is obtainable for long periods of time. A further advantage is that the actual substrate concentration in continuous culture is close to zero. This is of great importance in cultures with hydrocarbon substrates because cells adhere to emulsified substrate droplets thereby making

harvesting less efficient (Kappeli *et al* 1984). In the present study the batch fermenter was useful for optimising the defined codeine transformation system of *Candida tropicalis* ATCC 32113 and the amount of cytochrome P-450 produced by the cells. The *Candida tropicalis* ATCC 32113 cells were therefore useful in the subsequent cell free extract studies.

A procedure was developed to prepare cell free extracts from *Candida tropicalis* ATCC 32113, with a high degree of codeine N-demethylation activity and cytochrome P-450 content retained. The requirement of the cell wall lysing enzyme, lyticase (from *Arthrobacter luteus*) suggested that the active fraction was membrane bound. Ishidate *et al* (1963) first demonstrated that the cytochrome P-450 haemoprotein was bound to particulate fraction, the microsomes. The N-demethylation activity and the P-450 content of the cell free extract was enhanced by using a calcium chloride precipitation technique to produce cell free extracts. Although the cell free extracts were enriched for cytochrome P-450, attempts were not made in this study to investigate the mechanism of codeine N-demethylation by the cell free extracts. Carrying out enzyme kinetics experiments to obtain Michaelis Constants (Km) and the maximum velocity values may provide valuable information about the mechanism of N-demethylation. Such values obtained for transformation of codeine by *Cunninghamella* sp have shown that codeine N-demethylation does not occur *via* an N-oxide intermediate (Gibson, 1984). The 30% inhibition of N-demethylation activity by potassium cyanide in this study suggested that codeine N-demethylation may have been occuring at

least partially *via* an N-oxide. Further investigations need to be carried out on the ability of the enriched cell free extracts to demethylate codeine N-oxide. Such work in conjunction with the use of selective inhibitors such as potassium cyanide and kinetic experiments may provide a further insight into the N-demethylation mechanism.

The amount of codeine N-demethylated by enriched cell free extracts increased with codeine concentration up to 2mM. The significant fall in N-demethylation activity above 2mM was attributed to catabolite repression or denaturation of the cytochrome P-450 enzyme. The N-demethylation activity of the enriched cell free extracts was not increased by increasing the concentration of the co-factors NADPH, NADH and Fe^{2+} above 1mM. The activity detected in the absence of added co-factors suggested the presence of an endogenous supply of co-factors. The present studies suggested that the replenishment of the co-factors may be important for the N-demethylation reaction to be optimised. Although a co-factor regeneration system may be employed, this may not be viable on a larger scale. This may be overcome by immobilizing the microbial cells on an inert support. Whole cell immobilization also offers further advantages including regeneration and re-use of cells, increased catalytic density, increased cell stability and lower risks of contamination (Cheetham, 1981). The requirements of an enzyme extraction procedure and the problem of catabolis repression and substrate inhibition could also be avoided by whole cell immobilization. Cell immobilization systems have been utilized by

other workers. For example Schubert *et al* (1982) developed a system to entrap an active microsomal P-450 system in calcium alginate which was capable of N-demethylating aminopyrene and ethylmorphine. Further studies should therefore investigate the possibility of using alternative support materials for the imobilization of whole cells of *Candida tropicalis*. Inert materials such as polyacrylamide gels, collagen dispersions or polymeric metal hydroxide gels could be investigated.

Codeine N-demethylation activity was significantly inhibited by carbon monoxide and nitrogen gases. This suggested that the gas molecules were forming inert complexes with the cytochrome P-450 where the inhibition was irreversible. The drugs ketoconazole and miconazole also produced significant inhibition of codeine N-demethylation activity. These drugs are known to be specific cytochrome P-450 inhibitors (Higashi *et al*,1987). This was strong evidence for the possibility of cytochrome P-450 being the enzyme responsible for the codeine N-demethylation reaction. NMR spectroscopy was not used in this study but further experiments employing NMR may provide useful data about the nature of interaction between substrates and inhibitors with the cytochrome P-450 active site. A NMR method was used by Gibson (1984) to investigate the mechanism of codeine N-demethylation by *Cunninghamella bainer*i and such a procedure could be adopted for future interaction studies. This may be assisted by the findings in this study where enriched cell free extracts stored in air at -4°C retained a high degree of N-demethylation activity and cytochrome P-450 content up to periods

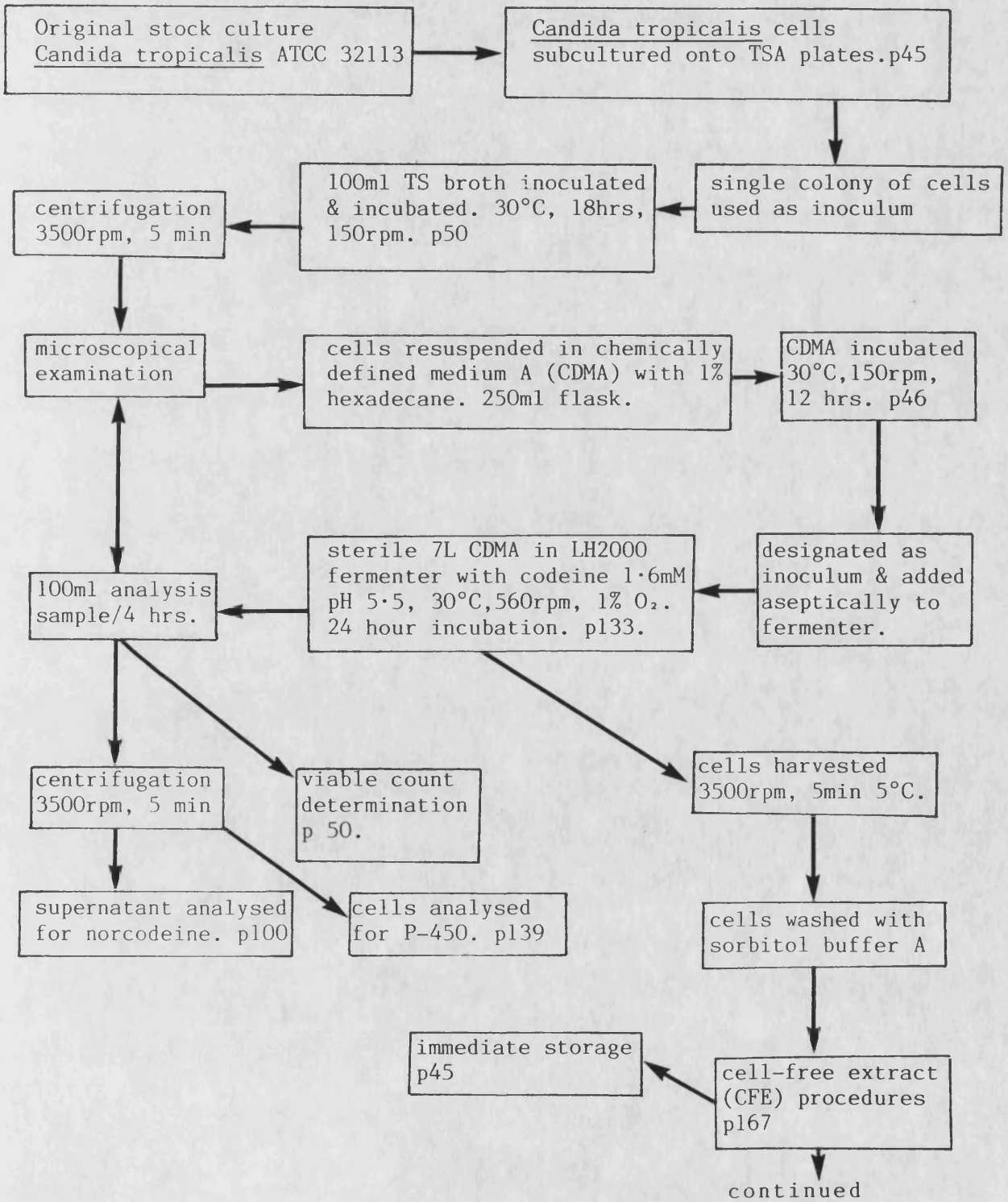
of 70 hours.

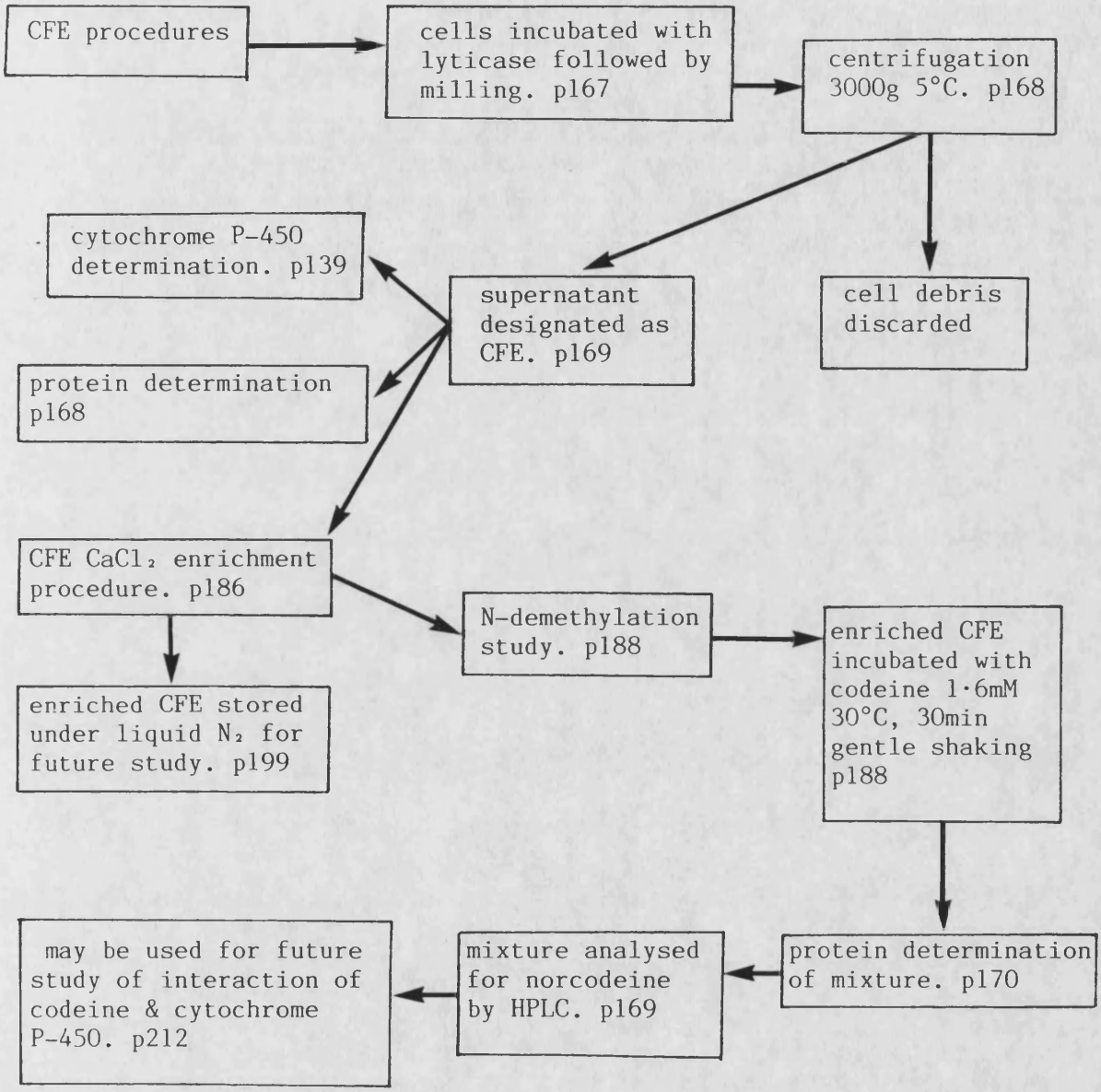
Microbial transformations of polar substrates with ionizable groups (such as codeine phosphate) could be improved by reacting the drug with an ion pair of the opposite charge. Upon ion pairing, the ionizable substrate will be closer to electrical neutrality affording greater lipid solubility. Tomlinson (1980) for example has shown that penetration of anhydrocarbonaceous polyamide 6-membranes by sodium chromoglycate is enhanced significantly by the presence of an organic ion of the opposite electrical charge.

The present study has produced a defined reproducible codeine N-demethylation system from *Candida tropicalis* ATCC 32113. This system therefore provides a basis for further experiments to be carried out to investigate and characterise the N-demethylation reaction further and screen other yeasts and strains of *Candida* for enhanced N-demethylation activity.

Cytochrome P-450 production process summary

To give a more complete overall picture of the 'process' this thesis describes, the following process diagram summarises the production of cytochrome P-450 from Candida tropicalis ATCC 32113 cells.




CFE procedures
cells incubated with lyticase followed by milling. p167
centrifugation 3000g 5°C. p168
cytochrome P-450 determination. p139
supernatant designated as CFE. p169
cell debris discarded
protein determination p168
CFE $CaCl_2$ enrichment procedure. p186
N-demethylation study. p188
enriched CFE incubated with codeine 1·6mM 30°C, 30min gentle shaking p188
enriched CFE stored under liquid N_2 for future study. p199
protein determination of mixture. p170
mixture analysed for norcodeine by HPLC. p169
may be used for future study of interaction of codeine & cytochrome P-450. p212

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REFERENCES

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